OXIDATION-REDUCTION POTENTIAL-BASED MICRO-AERATION CONTROL SYSTEM FOR ANAEROBIC DIGESTION

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To

Mom and Dad

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ABSTRACT

This study developed an intermittent oxidation-reduction potential (ORP)-controlled micro-aeration system for anaerobic digestion (AD) to avoid volatile fatty acids (VFA) accumulation at high organic loading rate (OLR). Without micro-aeration, AD of Napier grass, a typical energy crop, at an OLR of 5.0 g volatile solids (VS)/L/day resulted in a total VFA concentration up to 11.0 g/L as acetic acid, causing rapid drops in pH and methane yield regardless of pH adjustments, and driving the digester to the verge of failure. Once intermittent (every 24 h) ORP-controlled micro-aeration was introduced in 3 replicated studies, the average total VFA concentration decreased by 56% and the methane yield enhanced by 252%, resulting in stable performance without the need for chemical addition or OLR reduction. By combining reactor performance results, mass balance analyses, microbial community characterization data, and bioenergetics evaluations, this study suggested that an alternative pathway of VFA conversion could be accomplished through a synergistic linkage between anaerobic and aerobic conditions, bypassing syntrophic reactions typically found in anaerobic digesters. Meanwhile, intermittent ORP set at +25 mV from anaerobic baseline level preserved niches of anaerobic methanogens for effective methanogenesis. This novel operating approach can be applied as an effective process control strategy for the digestion of lignocellulosic biomass at high OLRs and offers significant economical and logistical merits.

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Chapter 1

Introduction

1.1 Background

Anaerobic digestion (AD), a process originally used for treating municipal and industrial wastewaters, was successfully adopted for bioenergy production using diverse feedstocks such as sewage sludge, municipal solid waste, food wastes, agricultural residues, animal manures and energy crops. The major advantages of AD process include low energy consumption, sequestration of greenhouse gases, and above all, its ability to remediate diverse organic wastes with concomitant production of renewable energy, biogas (Khanal and Li, 2016). The applications of biogas from AD plants for heat and electricity generation or upgrading into natural gas for injection into natural gas grid, have been widely practiced, especially in European countries, where there are over 17,376 commercial biogas plants currently in generating 8,293 MW of electricity equivalent (EBA, 2018). In the United States, there are currently 266 farm-based and 1,270 municipal sludge treatment-based anaerobic digesters in operation (EPA, 2018; WEF, 2018). However, 63 farm-based AD plants among total of 266 plants were shut down, and many other AD plants are on the verge of closing down mostly due to economic issue caused by the process instability and low biogas production (EPA, 2018).

Slow digestibility, especially when fed with recalcitrant lignocellulosic biomass, is one of the main reasons for low biogas production (Sawatdeenarunat et al., 2015). To overcome this limitation, digesters are commonly operated at high organic loading rates (OLRs) using substrates with a high total solids (TS) content. However, at high OLRs with high TS content, digesters are susceptible to failure due to accumulation of volatile fatty acids (VFA) (mainly acetic, propionic and butyric acids) caused by kinetic and energetic imbalance between fermentative acidogens, syntrophic acetogens and methanogens (Leng et al., 2017; Pind et al., 2003; Treu et al., 2016).

Automatic AD process control system enables quick process stabilization by closely

monitoring and controlling the process. First, the process parameters/indicators that are sensitive to process disturbances, such as pH, biogas production rate, VFA concentration, or total VFA to total alkalinity (VFA/ALK) ratio are closely monitored. The monitoring data is then sent to control system to regulate these parameters within the desired ranges either by adding chemicals to control the pH or regulating feeding rate using diverse process control systems (Nguyen et al., 2015). Stopping or reducing the substrate feeding rate allows microbes in the AD process to consume the accumulated metabolites and undigested substrates in the reactor and slowly overcome organic overloading condition. However, cessation of substrate feeding leads to piling-up of feedstock that needs to be digested and also reduces profits associated with biogas applications. Therefore, there is a critical need to find an alternative controlled strategy that allows reactor to stably operate at a higher OLR without the need of adding chemical or compromising the substrate feeding rate.

To this end, we developed a unique approach of improving the digester stability through intermittent injection of small amount of oxygen (micro-aeration), by controlling the oxidation-reduction potential (ORP) of the AD process. Recent studies have also shown that micro-aeration in anaerobic processes enhances hydrogen sulfide removal from biogas (Díaz et al., 2011b; Khanal and Huang, 2006; Krayzelova et al., 2015), facilitates hydrolysis (Lim and Wang, 2013; Xu et al., 2014a), enhances VFA production (Jagadabhi et al., 2010a; Sawatdeenarunat et al., 2017), and improves methane production (Nguyen et al., 2007, Lim and Wang, 2013). The beneficial effects of micro-aeration in AD processes is due to the augmentation in diversity and activity of facultative bacteria that promote hydrolysis, fermentation, and sulfide oxidation (Krayzelova et al., 2015; Lim et al., 2014; Zhu et al., 2009). Based on this reasoning, we hypothesize that VFA accumulated in the digesters during overloading conditions are rapidly consumed by facultative heterotrophs under micro-aerobic conditions thus contributing to AD process stability.

Few studies focused on an effective micro-aeration dosing system that can be applied to a mix-culture AD process or effect of micro-aeration on reducing VFA accumulation in the digester. To use micro-aeration as an effective operational strategy in AD process, precise

dosing control is needed to prevent oxygen overdose and inhibition of obligate anaerobes. ORP or redox potential, which exhibits a logarithmic relationship with dissolved oxygen (DO) concentration (See Appendix A), can be used for precise oxygen/air dosing control. This approach was previously applied in biological hydrogen sulfide removal (Khanal and Huang, 2003), biological nitrogen removal (Won and Ra, 2011), and pure culture fermentation processes to augment the product yields (Liu et al., 2013). However, insights into the effects of ORP-controlled micro-aeration on aerobic-anaerobic metabolic pathways and synergistic interactions between bacterial-archaeal populations in AD process are lacking.

1.2 Objectives of study

The overall goal of this study was to develop and validate the performance of an ORP-based micro-aeration system to control VFA accumulation and process stability without the inhibition of methanogenesis during AD of lignocellulosic feedstock at high OLR. The ORP-based micro-aeration was also examined for AD process stability control. By combining reactor performance and microbial community analyses with bioenergetics and mass balance calculations, we provided new insights into the dynamics of ORP-controlled micro-aeration in AD process and presented an effective strategy for maintaining the stability of a highly-loaded AD process.

The specific objectives are to:

1. Identify key monitoring parameters to evaluate the reactor performance at incremental organic loading rates.

2. Develop and validate the performance of an ORP-based micro-aeration process control system for maintaining stability of anaerobic digestion at high loading rate.

3. Evaluate the effect of micro-aeration on microbial community in anaerobic digestion processes.

1.3 Scope of the study

The study was performed in a semi-continuous lab-scale (2.0 L working volume) anaerobic bioreactor operated at mesophilic temperature (35^{o}C) and fed with Napier grass at OLR of 1.5, 3.0, and 5.0 g VS/L/day. The performance of the micro-aeration process control system was evaluated based on its ability to reduce VFA concentration and recover methane yield in triplicated experiments. Putative mechanism of the ORP-based micro-aeration system was proposed based on microbial community analyses with bioenergetics and mass balance calculations.

Chapter 2

Literature Review

2.1 Anaerobic digestion

2.1.1 Anaerobic digestion process

Anaerobic digestion (AD) processes have successfully been employed to remediate waste ranging from high strength industrial wastewaters, sewage sludge, municipal solids wastes, agricultural wastes/residues to animal manures while generating renewable energy. The major advantages of AD process, especially in waste (water) remediation include low energy consumption, less sludge yield, and above all, capability of stabilizing diverse organic wastes with concomitant production of renewable energy, biogas (Metcalf and Eddy, 2004; Khanal, 2008). In recent years, AD technology has been applied for generation of renewable energy and plethora of bio-based products (e.g., organic acids, biopolymer, bio-oil, biochar, etc.) by adopting AD biorefinery concept (Sawatdeenarunat et al., 2016; Surendra et al., 2015). The use of lignocellulosic biomass (e.g., agricultural residues and energy crops) as feedstock for AD biorefinery was recently examined due to the high yield with low inputs requirements (Sawatdeenarunat et al., 2015; 2017).

The fundamentals of AD process has been well documented in many books and reviews (Khanal, 2008; Vanwonterghem et al., 2014; Zitomer et al., 2016). AD is a complex, interrelated biological process involving diverse microbial communities supporting a series of interdependent biochemical reactions. As illustrated in Figure 2.1, the process starts with the hydrolysis of complex polymers into simple soluble products, followed by fermentation of simple soluble products into short-chain fatty acids (i.e., volatile fatty acids (VFA) with 2 to 7 carbons), CO_2, H_2, ethanol, lactic acids etc., and the process is known as acidogenesis. The anaerobic oxidation breaks down these VFA into acetic acid, CO_2, and H_2 via a process known as acetogenesis, and finally acetate, and $CO_2 + H_2$ is converted into methane via acetoclastic and hydrogenotrophic methanogenesis, respectively.

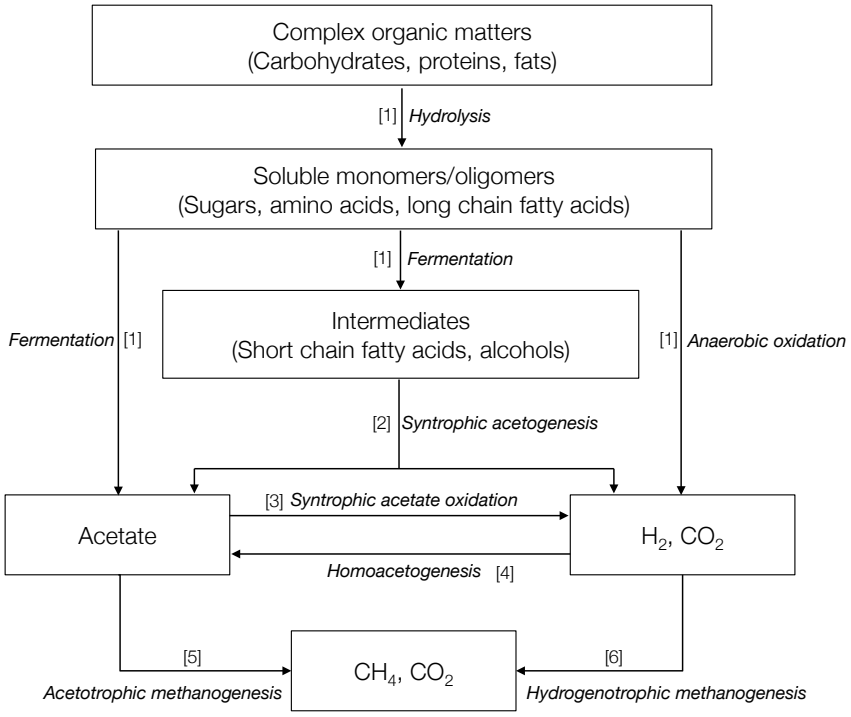

Figure 2.1 General pathway of anaerobic digestion processes

Note: Number represent functional microbial groups: [1] hydrolysis and fermentative bacteria; [2] syntrophic acetogens; [3] syntrophic acetate-oxidizing bacteria; [4] homoacetogens; [5] acetotrophic or aceticlastic methanogens; [6] hydrogenotrophic methanogens.

2.1.2 Limitations of anaerobic digestion process

Limitations of the AD process are rooted to intrinsic difficulty in maintaining a balance among the activities of hydrolytic, acidogenic, acetogenic, and methanogenic microorganisms. In a process where the product from one microbial group is the substrate for the next group, the kinetic and energetic balance between these biochemical reactions have to be in equilibrium. If such balance is not maintained, accumulations of intermediates is inevitable. For example, the VFA accumulation is commonly observed in AD processes due to its high production rate from fermentative bacteria as compared to low consumption rate of syntrophic acetogens. With hydrogen partial pressure (pH_2) as a limiting factor, syntrophic acetogens face thermodynamic constraint to convert VFA to

acetate, CO_2, and H_2 for the utilization by methanogens. This syntrophic relationship is extremely sensitive since pH_2 has to be low enough ($<10^{-4}$ atm) for the anaerobic degradation of VFA to be thermodynamically favorable, but high enough for the consumption by H_2-utilizing methanogens (Dolfing, 2014; Labib et al., 1993). Failure to maintain syntrophy within the microbiome may lead to irreversible AD process failure due to VFA build-up, which requires restart of the digester thereby causing significant economic loss (Dong et al., 2011; Steyer et al., 2006). As a result, many industries/clients are hesitant to implement AD technology despite its inherent advantages.

2.1.3 Anaerobic digestion of lignocellulosic biomass

AD has been widely adopted for digestion of lignocellulosic feedstocks to produce bioenergy, bio-based products, and concentrating nutrients in the digestate among others (Khanal and Li, 2016). Lignocellulosic feedstocks are the most abundant renewable resource on Earth, with an availability of approximately 200 billion dry metric tons per year (Ragauskas, 2006), distinguished from other feedstocks by their year-round availability with uniform composition, and relatively high yield, especially energy crops such as Napier grass (Sawatdeenarunat et al., 2015).

The AD of lignocellulosic biomass is illustrated in Figure 2.2. Lignocellulosic feedstocks are composed primarily of three polymers (hemicellulose, cellulose, and lignin) making it highly recalcitrant to biological degradation and causing low methane yield of AD reactors fed with this type of feedstocks (Sawatdeenarunat et al., 2015; Takara and Khanal, 2015). To overcome this limitation, digesters fed with lignocellulosic feedstock are normally operated at high OLRs with high total solid (TS) content for optimum resource utilization and biogas production. However, at high OLRs, digesters are susceptible to failure due to accumulations of VFA (mainly acetate and propionate) caused by kinetic imbalance between syntrophs and methanogens (Boe and Angelidaki, 2012; Ward et al., 2008).

Existing process control systems normally require stop feeding and/or addition of high amount of buffering chemical, without effectively resolve the origin of the instability from VFA accumulation, which is kinetic and energetic imbalance among microbial

consortia. Hence, there is a critical need to develop an effective strategy to promote the syntrophic growth of diverse microorganisms to maintain stability and rapid recovery of AD processes from potential failure due to VFA accumulation without supplementing chemical and compromising optimum OLRs.

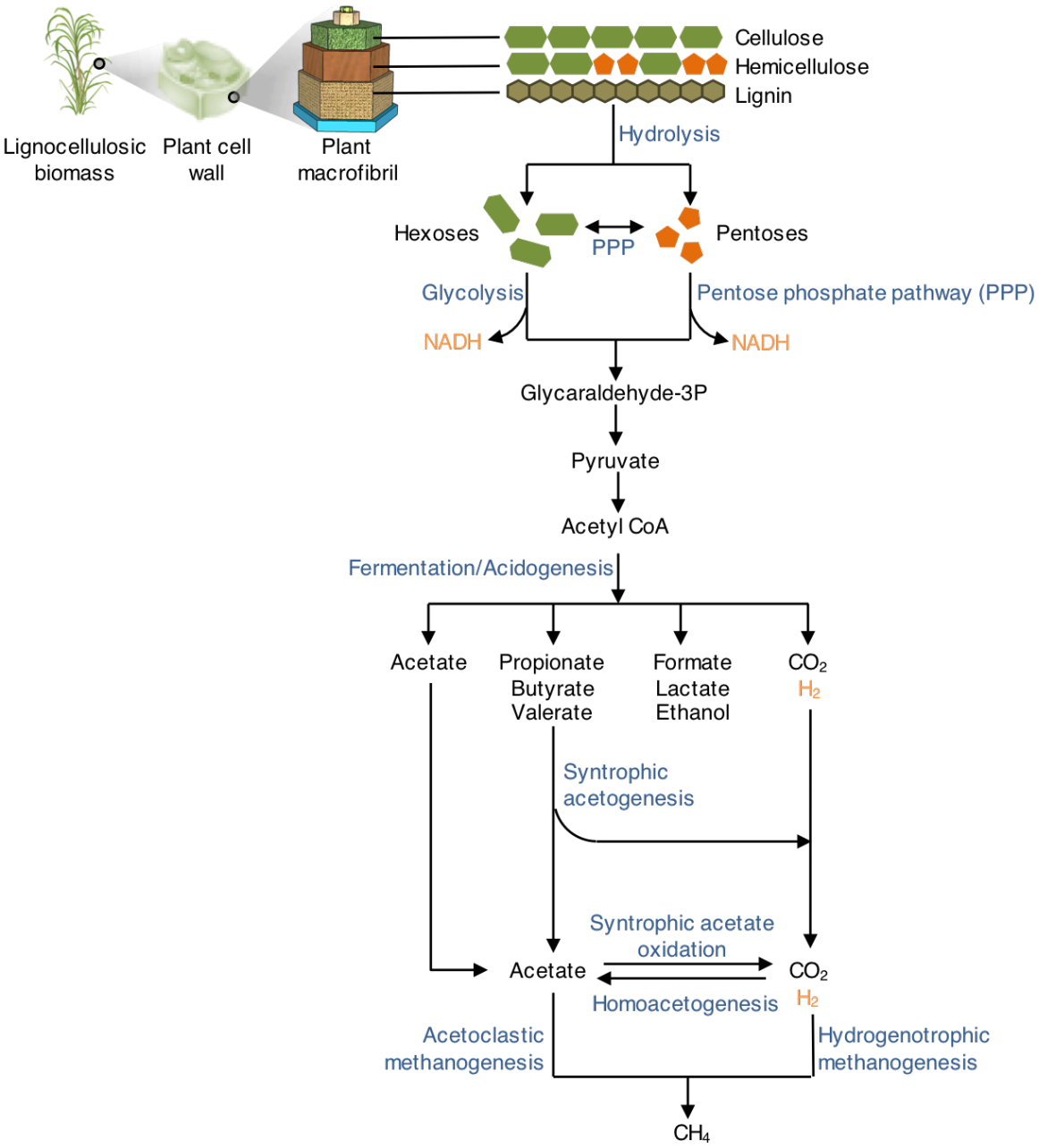

Figure 2.2 Pathway of the anaerobic digestion process of lignocellulosic biomass

Note: Adapted from Li and Khanal (2016) and Madigan et al. (2015).

2.2 Automatic monitoring and control of AD processes

Recent advancements in monitoring techniques and process control algorithms enables early identification of disturbances and rapid process stabilization, respectively. First, the process parameters that are sensitive to process disturbances, such as pH, biogas/methane production rate, ammonia, VFA composition, and total VFA to alkalinity (VFA/ALK) ratio are closely monitored. Next, the monitoring data is sent to control system to regulate these parameters around desired ranges. Diverse process control systems have been studied and tested in AD systems. The control strategy could be as simple as a feedback on/off control, or as complicated as adaptive, fuzzy logic, neural network control, or their combinations (Peter F Pind et al., 2003; Batstone et al., 2004; Drosg, 2013). As illustrated in Figure 2.3, advanced control systems were normally equipped with basic monitoring techniques; on the other hand, simple control strategies could be compensated by advanced monitoring equipment (Nguyen et al., 2015).

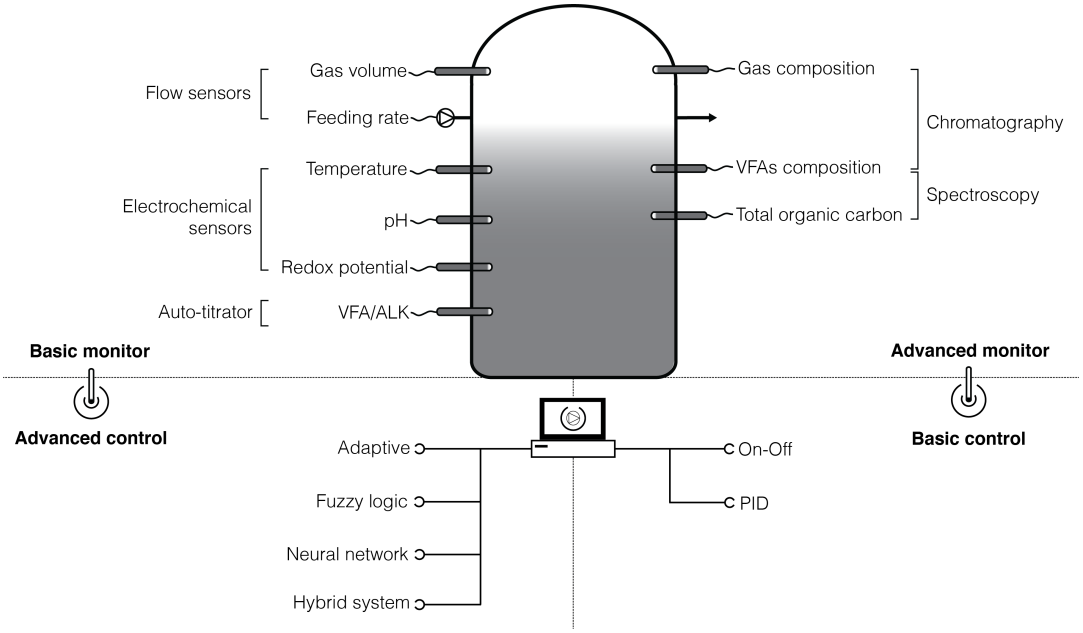

Figure 2.3 Typical automatic monitoring and control strategies in AD processes

2.2.1 Monitoring of AD processes

Process monitoring is the initial step and crucial component of any AD automatic control systems. Advances in instrumentation enable the on-line (real-time) monitoring of critical

parameters in the AD system for early detection of process disturbances. Such instruments should also be able to send early warning signal to operators or control algorithm in case of manual and automatic control, respectively, and ultimately promote the stable operation of AD process. Besides the fundamental operating parameters (e.g., substrate composition, biogas production and composition, pH, and temperature) of the AD process, the parameters indicating process disturbances (e.g. individual VFA, VFA/ALK ratio, ammonia and hydrogen) are of greater interest (Björnsson et al., 2000; Drosg, 2013). The process disturbances associated with an increasing organic loading rate are due to accumulation of intermediates, such as VFA, H_2, or ammonia during digestion (Batstone et al., 2004). Thus, it is critical to control these key parameters within their optimum ranges as presented in Table 2.1.

Table 2.1 Optimum operational range of the typical AD process

Parameters	**Mixed-culture**	**Hydrolysis/ Acidogenesis**	**Methanogenesis**
pH	6.8-7.4	5.2-6.3	6.7-7.5
Temperature (^oC)	Mesophilic: 35	Mesophilic: 25-35	Mesophilic: 32-42
	Thermophilic: 55	Thermophilic: 50-58	Thermophilic: 50-58
Solids retention time (days)	High-rate: 15-30		
	Low-rate: 30-60		
Total VFA (mg/L as acetic acid)	50-250		
Total alkalinity (mg/L as $CaCO_{3)}$	1,500-3,000		
Total VFA to total alkalinity (VFA/ALK) ratio	0.1-0.2		
Acetic acid (mg/L)	<1000		

Propionic acid (mg/L)	<250		
ORP (mV-E_h)	-200 to -350	+400 to -300	<-250
Carbon to nitrogen (C/N) ratio	20-30	10-45	20-30
Carbon to nitrogen to phosphorus (C:N:P) ratio	350:7:1	100:5:1	120:5:1
Total ammonia nitrogen (mg N/L)	50-1,000	≤ 1,500	
H_2 (ppm)	<100		

Sources: Khanal (2008), Deublein and Steinhauser (2011)

2.2.2 Automatic control of AD processes

Different from process control experiments carried out in laboratory or pilot-scale anaerobic reactor, industrial scale AD plants just control simple parameters, which include pH, temperature, mixed liquor level, gas pressure, mixed liquor and biogas flow rate (Vanrolleghem and Lee, 2003; Spanjers and Lier, 2006; Wiese and Haeck, 2006). Only 10% among 400 industrial scale anaerobic reactors worldwide are equipped with on-line analysis of COD, TOC, VFA, alkalinity, and biogas composition (Spanjers and Lier, 2006). This situation could be explained from the complexity in operation and maintenance of these advanced analyzers. Additionally, high capital and operation costs of these state-of-the art devices make it economically unattractive for biogas operators to embrace the technology.

The survey on manure-based biogas plants in Europe also revealed poor management of data monitoring and process control (Wiese and Haeck, 2006). In fact, continuous on-line monitoring was not performed in many plants. For plants with on-line monitoring systems, real-time control was rare and even the periodical data analysis was skipped. For the plants with real-time controllers, the control system was simple, time-based, equipped with on-off controller. The main reason for this poor state of automation is that the

numbers of these biogas plants are small-scale plants with electricity production capacity under 125 kW. Similar scenarios were reported in the United States, where most of the farm-based AD plants were shut down due to high maintenance cost and low biogas production (Beddoes et al., 2007). As a result, these small-scale AD plants cannot afford the expenses to acquire the instruments and automate the plant operation. This situation prompts that the centralized biogas plants may be a better solution to automate the AD process in a given geographical location. The investment cost for adequately instrumented and automated biogas plant with electricity generation capacity larger than 300 kW has been estimated to be just 5-10% of total capital cost (Wiese and Haeck, 2006). This investment can be profitable in long term operations, since a 10% drop in efficiency of a biogas plant can result in 11% decrease in the annual revenue (Wiese and Haeck, 2006). Even though the operational cost was not included in the report, this seems to be a worthy investment for large-scale industrial AD plants in long term.

Another problem of current AD process control system resides in ineffective manipulated input, which is typically feeding rate and/or pH adjustment (Figure 2.3). It means that when pH, VFA/ALK ratio, or methane production are out of optimum ranges, the process control system is activated to either stop feeding or start injecting chemicals to control pH of the digesters, or their combination. Adjusting pH only increases the buffering capacity to neutralize the digesters without solving the root of disturbance, which is VFA accumulation from kinetic and energetic imbalance. Stop feeding, in the other hand, leads to reduction in biogas production and accumulation of feedstocks. Besides, the cessation of feeding to AD digesters is not possible in some cases, for instance the sewage sludge digestion in wastewater treatment plants, since it affects the continuality of the whole process. As a result, alternative control input to control stability of digesters without requiring chemical addition or compensating optimum organic loading rate are much needed for effective process control of AD processes.

2.3 Micro-aeration in AD processes

2.3.1 Micro-aerobic environment

Micro-aeration is the dosing of small amount of air or oxygen into an anaerobic system. Different homologous terminologies are being used, namely micro-aeration, limited aeration, micro-oxygenation, oxygenation, and microaerobic condition among others (Krayzelova et al., 2015). Air or oxygen could be dosed either one time, intermittently (pulse-mode) or continuously at different stages of the AD process (pretreatment, during digestion or post digestion). As a result, the terminology "micro-aeration" has been vaguely defined due to lack of standard method to precisely monitor oxygen level in the AD system. In aerobic processes such as aerobic fermentation or activated sludge process, DO – residual oxygen concentration in aqueous phase – is used as a parameter to quantify aerobic condition. However, with the detection limit of 0.1 mg/L (3μM), DO probe lacks sensitivity needed to precisely quantify microaerobic environment in an AD system. Advanced nanomolar oxygen sensing technique has been developed in recent years to measure low oxygen concentration in biofilm or deep-sea sediment; but its industrial applications remain limited due to high cost and lack of robustness (Morris and Schmidt, 2013). The most practical and sound approach to standardize the microaerobic condition among the reported studies, is to use redox potential or oxidation-reduction potential (ORP). Since ORP varies linearly with the logarithmic of oxygen concentration, the ORP electrode can precisely sense very small changes in oxygen concentration in the aqueous phase (Khanal and Huang, 2006). For example, according to the correlation of ORP (E_{Ag} – ORP with reference to Ag/AgCl electrode) and DO in Figure A.3, DO of 0.1 mg/L is equivalent with ORP of -96 mV; anaerobic condition with ORP of -495 mV has an equivalent DO of 1.15 x 10^{-8} mg/L; and a 25 mV increase in ORP is equivalent to a DO increase of 0.02 x 10^{-6} mg/L or 1.25 x10^{-6} μM O_2.. Details about definition, measurement, and factors affecting ORP are presented in Appendix A.

Although ORP value is slightly affected by temperature (Figure A.1) and pH (Figure A.2), it can be used as parameter indicating oxic or anoxic environment. A microaerobic condition may be defined as an aqueous media with ORP (E_h – ORP with reference to standard hydrogen electrode (SHE)) value in the range of 0 to -300 mV. Anaerobic

systems have an average ORP level of -300 mV or lower (Khanal and Huang, 2006; Krayzelova et al., 2015). The ORP values of greater than 0 mV represent oxidizing environment of the aerobic condition (Figure 2.4).

Facultative microorganisms grow under anoxic or oxic condition with some strains can produce energy from aerobic respiration at a nanomolar O_2 concentration by having high affinity oxidase enzymes (Morris and Schmidt, 2013). Although aerotolerant anaerobes and obligate anaerobes are not considered facultatives, they co-exist and co-metabolite with facultatives under microaerobic condition with various antioxidative mechanisms, which are discussed in the later section. As depicted in Figure 2.4, facultatives grow optimally and produce fermentation products like ethanol, butanol and VFA within the ORP range of 0 to -300 mV. In addition, the common redox couples of reactions in AD processes such as SO_4^{2-}/HS^- (sulfidogenesis) or CO_2/CH_4 (methanogenesis) also have standard redox potential ($E^{o'}$) in this microaerobic range of -200 to -300 mV. The dosing of air or oxygen to an anaerobic process elevates the ORP to a more positive value, which then slowly decreases with the consumption of injected O_2 by facultative bacteria. Several studies employed ORP as the controlling parameter for micro-aeration to enhance VFA production (ORP of -100 to -200 mV) (Yin et al., 2016), sulfide removal (ORP of -275 to -265 mV) (Khanal et al., 2003), and to optimize fermentation process for ethanol, butanol, and propanediol production (ORP of -50 to -350 mV) (Liu et al., 2013). By manipulating the extracellular ORP of the environment, intracellular metabolic pathway of microbes (anaerobic oxidation, fermentation, or aerobic oxidation) can be modified at the molecular levels via electron flow, reducing power (i.e. NADH concentration), and gene expression (Liu et al., 2013). As a result, ORP could be used as an effective parameter for monitoring and controlling the micro-aeration in AD system.

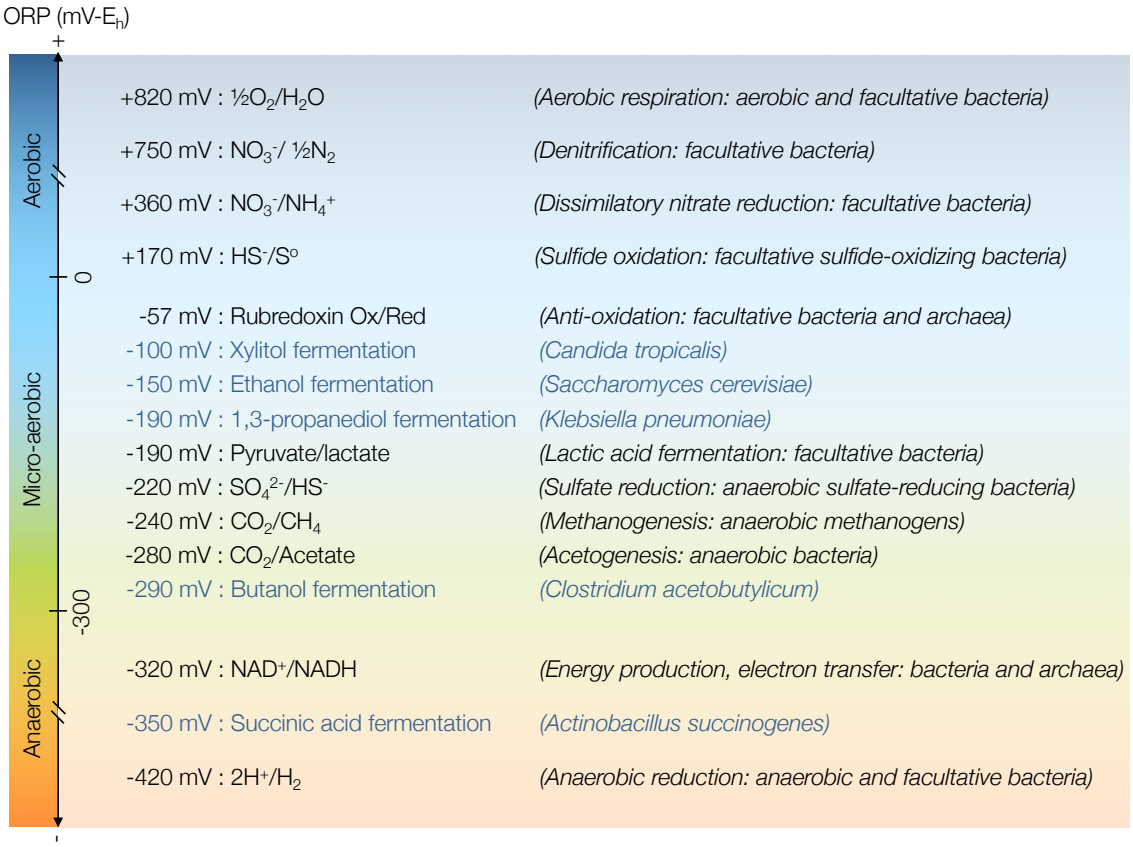

Figure 2.4 Standard redox potential of redox couples and optimum ORP of various microorganisms

Note: Position of data are not to scale with axis. Values in black text from Madigan et al., (2015) and blue text from Liu et al. (2013).

2.3.2 Mechanisms of oxygen tolerance in micro-aerobic environment

In micro-aerobic environment, facultatives (hydrolytic and fermentative bacteria) consume and partially reduced the oxygen molecule, generating reactive oxygen species (ROS) (i.e., O_2^-, H_2O_2, and •OH) (Figure 2.5). These active radicals and ions are highly oxidative and can damage lipid membrane, protein and DNA of microorganisms (Ezraty et al., 2017). Aerobic and facultative bacteria can produce anti-oxidative enzymes and neutralize these oxidative species, allowing them to thrive in aerobic conditions (Fu et al., 2015; Imlay, 2013). As shown in Figure 2.5, common anti-oxidative enzymes are superoxide dismutase (SOD); catalase; superoxide reductase (SOR) or rubredoxin oxidoreductase; and alkyl hydroperoxide reductase (Ahp).

Under an aerobic condition, several strict anaerobes could produce anti-oxidative enzymes to certain extent to adapt to the oxidative environment (Brioukhanov et al., 2006). For example, under an oxidative stress, anaerobes *Clostridium perfringens, Clostridium acetobutylicum, Bacteroides fragilis, Desulfovibrio gigas, Methanosarcina barkeri, Methanobacterium* and *Methanobrevibacter* showed an overexpression of genes encoding SOD, catalase, and superoxide reductate enzymes (Brioukhanov et al., 2006; Horne and Lessner, 2013). Recent study also shown methanogens can also survived in aerobic fresh water and soil with O_2 concentration up to 10%, indicating an effective oxygen tolerance of strict anaerobes (Angle et al., 2017). Such adaptive responses of strict anaerobic bacteria and archaea allow substantial survival rate of these microorganisms in the micro-aerobic conditions.

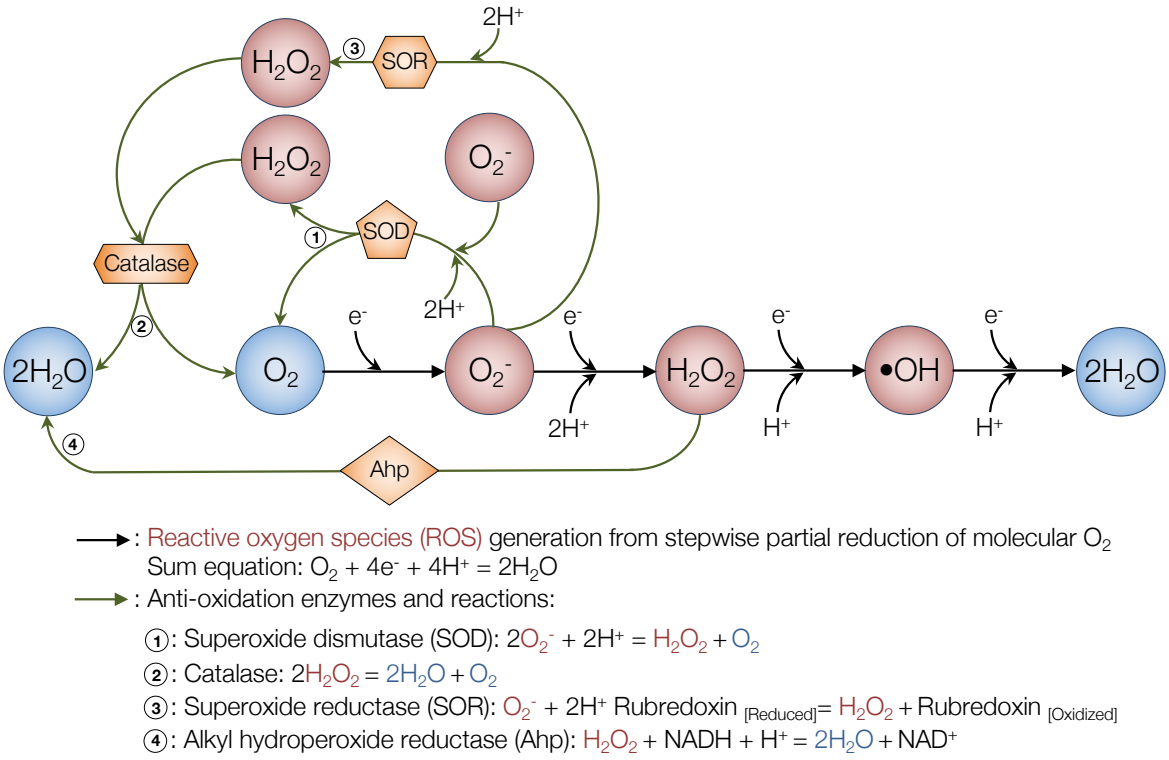

Figure 2.5 Reactive oxygen species generation and anti-oxidative stress mechanism of microorganism

Co-existence and synergy interaction between facultative and anaerobic microorganisms in AD process is another mechanism for oxygen tolerance ability of strict anaerobes that cannot produce anti-oxidative enzymes. In AD process, microbial consortia tend to form flocs with strict anaerobes in the center and facultative bacteria in the outer layer (Botheju and Bakke, 2011). Facultative bacteria (i.e., hydrolytic and fermentative bacteria) with higher anti-oxidative enzyme activity could scavenge ROS and protect the anaerobes, which are more susceptible to oxygen exposure (Figure 2.6). In addition, the gradient of oxygen and ROS reduce as it diffuse through biofilm-like layer and is consumed/converted completely before reaching and damaging the inner anaerobic microorganisms (Stewart and Franklin, 2008). As depicted in Figure 2.6a, the synergetic relationship is also shown in substrate flow from outer to inner section of the aggregated floc, where hydrolytic bacteria can consume oxygen to effectively breakdown complex organic matters into various intermediates that serve as substrates for fermentative bacteria and then anaerobic methanogens. As a result, the residual oxygen, which can be measured by ORP, declines to the level that is more tolerable to strict anaerobes (Figure 2.6c). Hence, this outer layer of facultative bacteria acts as both physical and biological oxygen shield for strict anaerobic bacteria and archaea, allowing them to survive in micro-aerobic condition.

Insufficient oxygen dosing rate, besides not bringing significant augmentations, can cause adverse effects since it alters the balance of microbial community and require microbial adjustment to the new condition (Zhu et al., 2009). In the other hand, over-aeration beyond the oxygen consumption and anti-oxidation capacity of facultative bacteria can cause detrimental effects on strict anaerobes due to high concentration of free oxygen and ROS as well as aerobically oxidizes available substrates that eventually lead to reduced methane yield (Xu et al., 2014b) (Figure 2.6b). To supply sufficient amount of oxygen, various factors affecting oxygen transfer and utilization rate need to be considered, which are elucidated in section 2.3.4.

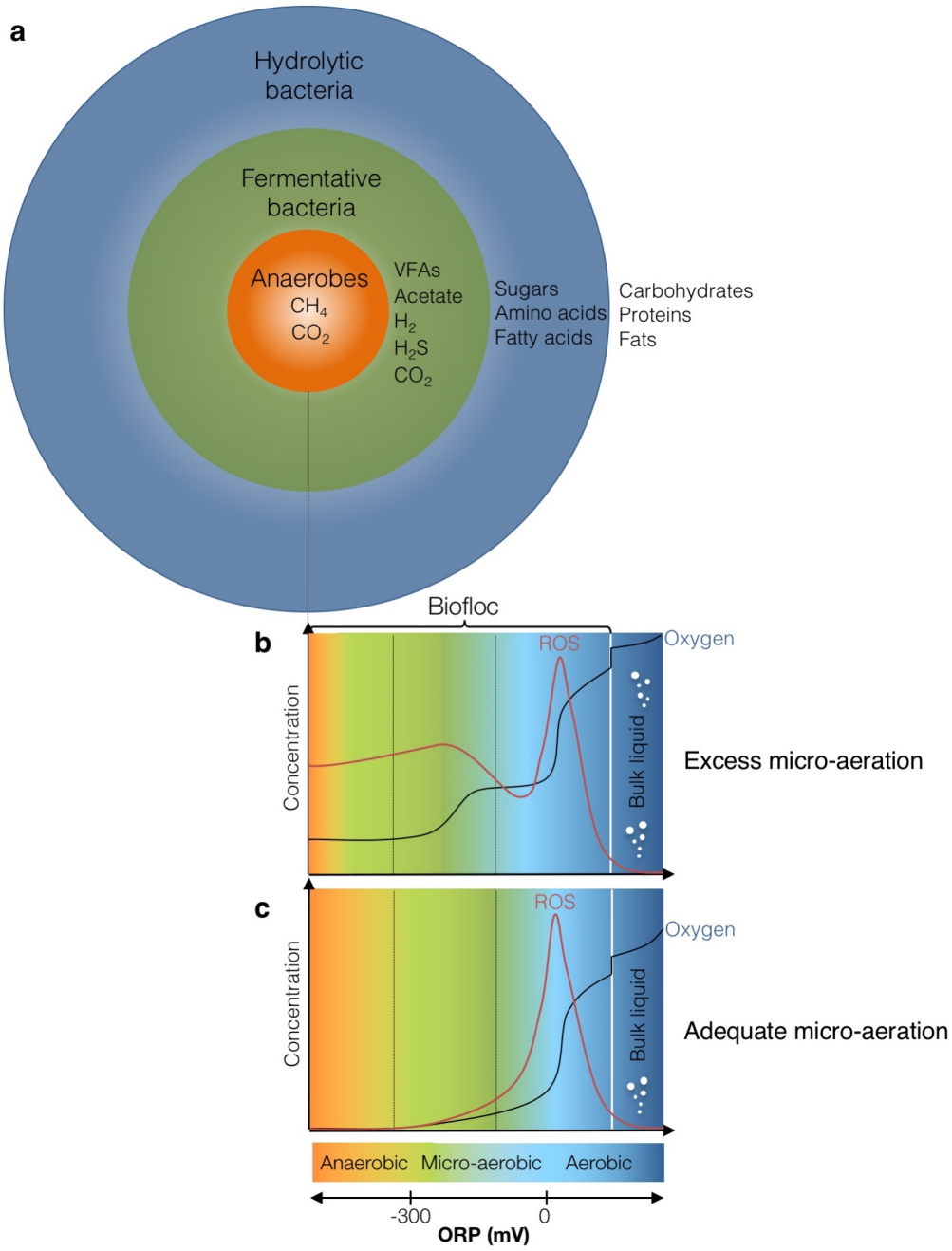

Figure 2.6 Anti-oxidative stress mechanisms of microbial community in micro-aerobic environment.

Note: Distribution of various microbial groups in bioflocs: strict anaerobes aggregate in the center, hydrolytic bacteria locate at the outer, and fermentative bacteria spread across the biofloc (**a**). Substrate gradient across bioflocs where products from the outer group are substrate of the adjunct inner group. Gradient of oxygen and ROS across the bioflocs in excess (**b**) and adequate micro-aeration intensity (**c**).

2.3.3 Differences between anaerobic and aerobic metabolic pathway

Micro-aeration-based AD processes create a unique environment that overlaps between anaerobic and aerobic conditions by maintaining niches for both anaerobes and micro-aerobes in such environment.

Under anaerobic condition, organic matters are partially reduced to energy-rich compounds (consist of phosphate bond or coenzyme A (CoA) molecule), which generate ATP via substrate level phosphorylation. Due to the lack of an effective mechanism to transport electrons, anaerobes produce less energy from the organic degradation reactions (4 mols ATP/mol glucose) (Figure 2.7). In addition, to maintain the redox balance (ratio of NADH/NAD^+) under an anaerobic condition, fermentative products (e.g. ethanol, lactate, and VFA) are produced to regenerate NAD^+ from NADH (Madigan et al., 2015). These intermediates are further converted to CH_4 via syntrophic oxidation reactions coupled with methanogenesis while generating minimal amount of energy using ion pumps to create proton or sodium motive force for ATP generation (Figure 2.7).

Under aerobic condition, on the other hand, acetyl-CoA goes through tricarboxylic acid (TCA) cycle to be completely oxidized to CO_2 through highly energetic reaction. Using O_2 as a terminal electron acceptor, aerobic oxidation of 1 mol glucose generates total of 32 mol ATP via glycolysis, TCA cycle and oxidative phosphorylation of reduced coenzymes (NADH and $FADH_2$) (Figure 2.7). With this highly energetic metabolism, aerobic microbes rapidly consume organic matters and grow faster than anaerobes, as evident from higher maximum specific growth rate, substrate affinity, and biomass yield (Table 2.3). Table 2.2 shows the comparison between energy produced from different VFA using aerobic respiration and syntrophic anaerobic oxidation. Thermodynamic constraint of syntrophic oxidation of VFA to acetate and H_2 is a bottleneck in AD processes leading to VFA accumulation and process instability, especially at high OLRs. Therefore, microaerobic condition integrating the aerobic VFA oxidation by heterotrophs with anaerobic methanogenesis could be a promising strategy to facilitate energetic conversions of intermediates to maintain overall stability of AD processes. To couple aerobic oxidation with anaerobic reduction reactions, effective micro-aeration control strategy is needed to prevent inhibition of obligate anaerobes, as previously discussed.

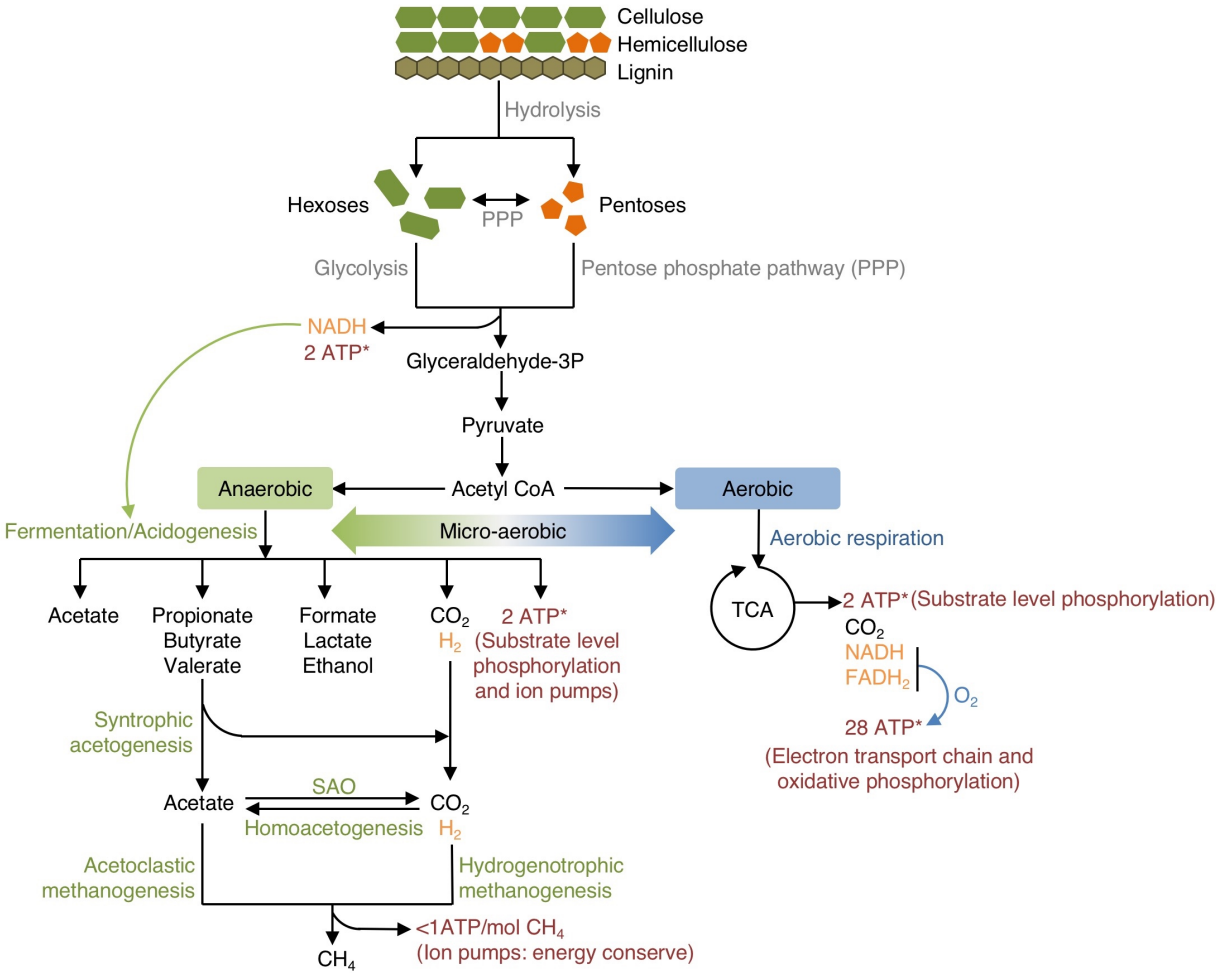

Figure 2.7 Metabolic pathway and energy production from lignocellulosic substrate in anaerobic and micro-aerobic environments.

Note: *ATPs produce from1 mol Glucose. SAO: syntrophic acetate oxidation. TCA: tricarboxylic acid cycle (i.e. citric acid cycle or Krebs cycle). Data from Madigan et al., (2015).

Table 2.2 Energetics of VFA consumption reactions under micro-aerobic and anaerobic condition

No.	Reactions	$\Delta G^{o'}$ (kJ)*	Electrons transferred	$\Delta G^{o'}$ (kJ/e-)
Anaerobic condition (Redox couple $2H^+/H_2$: $E^{o'}$= - 420 mV)				
1A	Valerate + 3 H_2O = 2.5 Acetate + 3 H_2 + 1.5 H^+	72.6	6	12.1
2A	Butyrate + 2 H_2O = 2 Acetate + 2 H_2 + H^+	48.3	4	12.1
3A	Propionate + 1 H_2O = 1.5 Acetate + H_2 + 0.5 H^+	24.2	2	12.1
4A	Acetate + H^+ + 2 H_2O = 2 CO_2 + 4 H_2	94.9	8	11.9
Micro-aerobic condition (Redox couple $0.5O_2/H_2O$: $E^{o'}$= + 820 mV)				
1B	Valerate + H^+ + 6.5 O_2 = 5 CO_2 + 5 H_2O	-2773.6	26	-106.7
2B	Butyrate + H^+ + 5 O_2 = 4 CO_2 + 4 H_2O	-2133.8	20	-106.7
3B	Propionate + H^+ + 3.5 O_2 = 3 CO_2 + 3 H_2O	-1493.8	14	-106.7
4B	Acetate + H^+ + 2 O_2 = 2 CO_2 + 2 H_2O	-853.9	8	-106.7
Methanogenesis (Redox couple CO_2/CH_4: $E^{o'}$= -240 mV)				
AM	Acetate + H^+ = CO_2 + CH_4	-35.9	8	-4.5
HM	CO_2 + 4 H_2 = 2 H_2O + CH_4	-130.8	8	-16.4

Note: *Standard Gibb's free energy ($\Delta G^{o'}$) is calculated with all gases at 1 atm, VFA and H_2O at 1 M, temperature of 25^oC, and at pH 7 (Dolfing, 2015; Thauer et al., 1977). Values of $\Delta G^{o'}$ change when use HCO_3^- + H^+ instead of CO_2 + H_2O for balancing reactions.

Table 2.3 Biokinetics of bacterial and archaeal groups

	μ_m	K_S	q_m	Y	Reference
Aerobic heterotrophic bacteria	13.20	20	27	0.49	(Rittmann and McCarty, 2001)
Carbohydrate fermentative bacteria	1.20	500	9.8	0.13	(Ni et al., 2015)
Anaerobic acetate degrader	0.38	165	9.5*	0.04	(Lawrence and McCarty, 1969)
Anaerobic propionate degrader	0.31	60	7.8*	0.04	(Lawrence and McCarty, 1969)
Anaerobic butyrate degrader	0.35	13	7.0*	0.05	(Lawrence and McCarty, 1969)
Sulfide-oxidizing bacteria	1.4	-	5[a]	0.28[c]	(Rittmann and McCarty, 2001)
Sulfate-reducing bacteria	0.29	-	1.05[b]	0.28[d]	(Rittmann and McCarty, 2001)
Acetoclastic methanogens	0.30	-	8.4	0.035	(Rittmann and McCarty, 2001)
Hydrogenotrophic methanogens	0.50	-	1.1[b]	0.45[d]	(Rittmann and McCarty, 2001)

Note: μ_m: maximum specific growth rate (1/day), K_S: half-velocity constant (mg COD/L), q_m: maximum specific substrate utilization rate (g COD/g VSS/day), Y: biomass yield (g VSS/g COD). *Calculate from equation $\mu_m = q_m * Y$ (Rittmann and McCarty, 2001). Specific unit: [a] g S/g VSS/day, [b] g H_2/g VSS/day, [c] g VSS/ g S, [d] g VSS/g H_2.

2.3.4 Factors affect micro-aeration intensity

Micro-aeration intensity is the main factor determining effects of micro-aeration on the AD system. The rate of micro-aeration varies depending on specific purpose, ranging from controlling VFA accumulation, stabilizing the process, removing hydrogen sulfide, increasing methane yield, enhancing hydrolysis, to improving VFA production (Figure 2.8). For example, Zhou et al., (2007) used the aeration rate of 0.38 mL O_2/mg S and

Krayzelova et al., (2015) used the molar ratio of O_2/S^{2-} of 0.5 to remove hydrogen sulfide from biogas. For enhancing hydrolysis, aeration intensity is usually calculated based on total solids (TS) content of the feedstock and is reported as L air/ kg TS.day (Nguyen et al., 2007; Zhu et al., 2009). Botheju et al. (2010), in other approach, used aeration rate of 2.5 to 10 % of COD of feedstock for improving the hydrolysis.

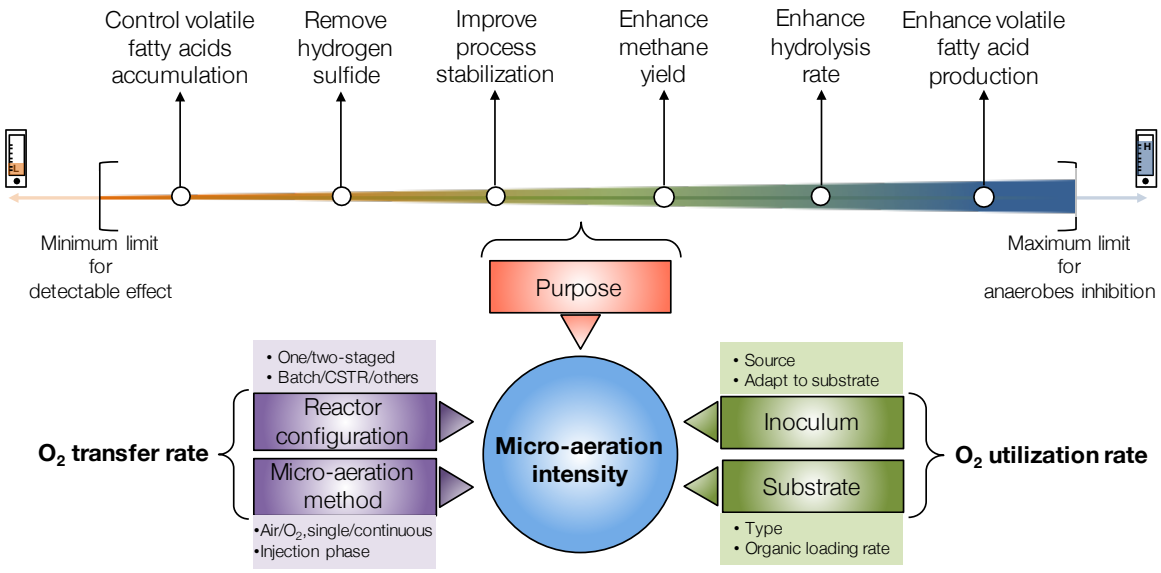

Figure 2.8 Factors determine effects of micro-aeration on anaerobic digestion

Despite the fact that micro-aeration rates could be determined based on specific purposes, oxygen transfer rate (OTR) and oxygen utilization rate (OUR) has to be considered to provide sufficient amount of oxygen in different situations. Micro-aeration systems follow oxygen mass balance principle showed in the equation below.

Accumulated O_2 = O_2 transfer rate – O_2 utilization rate = $(k_La * \Delta CO_2) – (qO_2 * C_x)$

Where, k_La is oxygen transfer coefficient, ΔC_{O2} is oxygen concentration gradient (= saturated oxygen concentration – dissolved oxygen concentration in liquid phase), q_{O2} is oxygen consumption rate of microbes, and C_x is microbial biomass concentration.

The equation explains that the micro-aeration intensity should be increased in a system that has low OTR such as in high solid loading rate AD process or in a system with high OUR from highly active aerobes or facultatives.

The OTR in the AD process depends on the factors such as reactor configuration, micro-

aeration method (i.e. use air or oxygen, air bubble size, exposure time in liquid, injection in liquid or gas phase), TS content of the reactor, and etc. (Garcia-ochoa and Gomez, 2009). The OUR could be determined based on concentration and characteristics of inoculum and substrate. OUR could also affect oxygen transfer rate by influencing the oxygen concentration gradient (ΔC_{O2}). Garcia-ochoa and Gomez (2009) observed a higher oxygen transfer rate in the AD system with a higher oxygen consumption rate. Understanding the dynamic and using k_La and OTR in micro-aerobic condition, we can scale-up the process while maintaining the same efficiency of the system at lab-scale (Fernández-Sandoval et al., 2017). The only mathematical tool available now to model the effect of oxygen in anaerobic process is the ADM1-Ox – an adapted model from the well-known Anaerobic Digestion Model No. 1 (ADM1) (Botheju et al., 2010). However, dynamic of OTR and OUR in micro-aerobic environment are yet to be studied and remained as interesting topic to be critically investigate in future researches.

The point of micro-aeration (i.e. liquid or gas phase; before or during AD) needs to be carefully considered depending on different micro-aeration purpose (Table 2.4). For example, injection of air or oxygen in the reactor headspace at high intensity was widely practiced for removing hydrogen sulfide with removal efficiency of as high as 99% (Krayzelova et al., 2015). However, due to the limited air diffusion rate between gas-liquid interphase especially in the high solids conditions, this micro-aeration method fails to impact processes in the liquid phase such as remove dissolved sulfate, sulphide, or improve hydrolysis rate (Boe et al., 2010; Sheets et al., 2015). Micro-aeration can be injected to inoculum tank, hydrolytic reactor of a two-staged reactor configuration, mix-cultured AD reactor, biogas or sludge recycled line, or digestate storage tank to achieve different purposes (Girotto et al., 2016). In the two-staged reactor configuration, when the hydrolytic reactor is micro-aerated, the risk of inhibiting methanogens would be minimized (Xu et al., 2014b). However, in single-staged reactor configuration, facultative bacteria were reported to quickly consume additional oxygen and protect the oxygen-sensitive methanogens (Botheju and Bakke, 2011). Under micro-aerobic condition, specific activities of methanogens were even improved as compared to that of the anaerobic condition due to diverse and well-balanced microbial community structure, resulting in low VFA accumulation and stable pH (Fu et al., 2016; Lim et al., 2014).

Characteristic and concentration of inoculum and substrate are crucial in determining the OUR in micro-aerobic condition. Microbial biomass concentration and microbial community structure in micro-aerobic digestion processes affect micro-aeration rate, and vice versa. In recent studies, micro-aeration was applied to increase the concentrations of microbial biomass that resulted in a shorter and more stable start-up time of anaerobic reactors (Díaz et al., 2011a; Jenicek et al., 2011). Therefore, micro-aeration treatment could mitigate the inhibitory effects of hydraulic overloading that caused biomass washed-out and rapidly recover the system under such overloading condition (Ramos and Fdz-Polanco, 2013). Taxonomic analysis in full-scaled AD reactors showed that *Bacteria* and *Archaea* domain consisted of 93% and 5.6 % of total DNA sequences, respectively, (J. Guo et al., 2015). Among bacteria, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* were dominant phyla. Similar result was observed in the studies by (Lim et al., 2013; Niu et al., 2015). The microbial structure also varied depending on inoculum type (Yin et al., 2016), reactor configuration (Lim et al., 2013), operation state (i.e. startup or stable phase) (Goux et al., 2016), substrate types (Hanreich et al., 2013) and micro-aeration treatment (Lim et al., 2014).

Substrate characteristics and concentrations affect the micro-aeration rate through hydrolysability and organic loading rate, respectively (

Table 2.4). AD reactors with substrate with recalcitrant feedstocks such as lignocellulosic biomass could use micro-aeration as biological pretreatment to enhance hydrolysis rate (Díaz et al., 2011a; Jagadabhi et al., 2010b). Micro-aeration intensity for substrates that have higher hydrolysis rate, such as food waste, wastewater, and sludge, require a fine adjustment to prevent the excess accumulation of VFA in the system while achieve the goal of improve methane yield (Johansen and Bakke, 2006; Lim and Wang, 2013; Xu et al., 2014b). In all applications of micro-aeration, substrate competition between facultative bacteria and anaerobic methanogens needs to be meticulously considered to maintain the balance of the enhanced digestion system and prevent the overall reduction in methane yield. Based on the purpose of the micro-aeration,

Table 2.4 could be used along with Figure 2.8 as guidance for future studies to decide the micro-aeration rate at the specific reactor conditions.

Table 2.4 Factors determining micro-aeration rate

Factors	Recommended micro-aeration purpose	Recommended micro-aeration rate*
Reactor configuration		
CSTR	Control VFA accumulation Sulfide removal	Low
UASB	Enhance methane production Sulfide removal	Medium
Leached bed reactor	Enhance hydrolysis	High
Acid tank of two-staged reactor	Enhance VFA production Enhance hydrolysis	High
Micro-aeration method		
Gas phase injection	H_2S gas removal	High
Liquid phase injection	Enhance hydrolysis Dissolved sulfide removal Enhance VFA production Control VFA accumulation	Low-Medium
Single injection	Enhance hydrolysis Enhance VFA production	High
Intermittent/continuous injection	Control VFA accumulation	Low
Inoculum		
Low biomass concentration	Reduce start-up time	Low
High biomass concentration	Control VFA accumulation Enhance hydrolysis	High
Low biomass diversity	Enhance hydrolysis Control VFA accumulation	High

High biomass diversity	-	Low
Substrate		
Low hydrolysability rate	Enhance hydrolysis	High
High hydrolysability rate	Enhance VFA production	Medium
	Enhance methane yield	
Low organic loading rate	Enhance hydrolysis	Low
High organic loading rate	Control VFA accumulation	High

Note: *Recommended micro-aeration rate based on previous studies: Low: 0.005-0.01, medium: 0.01-0.2, high: 0.2-5.0 L O_2/ L reactor/day (Table 2.5).

Table 2.5 Micro-aeration rate of various applications

Objective	Reactor configuration and working volume	Substrate	Micro-aeration intensity	Dosing method	O_2 dosing rate equivalent (L O_2/L/d)*	Result	Referenc
Enhance hydrolysis	CSTR (3L)	Food waste and brown water	5 mL and 7 mL O_2/L/d	Single dosing	0.005 and 0.007	More diverse bacterial population and higher VFA concentration	(Lim et al., 2014)
Enhance hydrolysis	CSTR (0.25L)	Food waste and brown water	0.037 L O_2/L/d	Daily dosing (4 days)	0.04	Enhance hydrolysis and acidogenesis	(Lim and Wang, 2013)
Enhance hydrolysis	CSTR (0.5L)	Primary sludge	500 ml air/d at flowrate 15 sec/min	Semi-continuous time based	0.21	50-60% enhance hydrolysis. Reduced methane yield, VFA and sCOD due to aerobic substrate consumption	(Johansen and Bakke, 2006)
Enhance hydrolysis	CSTR (1.6L)	Primary sludge	2.5 mL air/min	Continuous	0.5	Increase solubilization of COD by hydrolysis of carbohydrates and protein	(Diak et al., 2013)
Enhance hydrolysis	CSTR (2.5L)	Waste activated sludge	0.4 vvm and 0.08 vvm of air	Continuous	24-121	Increase solubilization	(Hasegawa et al., 2000)
Enhance hydrolysis	Leach bed reactor (4.6L)	Synthetic food waste	129, 258, 387 L air/kgTS/d at flow rate of 1L/min	Semi-continuous time based	2.1, 4.4, and 6.5	Middle aeration rate is best: increased hydrolysis and no effect on methanogenic reactor	(Xu et al., 2014b)

Enhance hydrolysis and acidification	Leach bed reactor (260L)	Municipal solid waste	1L air/min for 2h and stop 4h. 3.2 L air/kgTS/d	Semi-continuous time based	0.39	No significant increase of hydrolysis	(Nguyen et al., 2007)
Enhance methane yield	Batch (0.2L)	Corn straw	12.5, 25, 50, 100 mL air/L/d	Single	0.003-0.021	Enhance methane yield and VS removal at lower micro-aeration intensity. Enhance diversity of phylum *Firmicutes*	(Fu et al., 2016)
H_2S removal	CSTR (200L)	Waste activated sludge	0.013-0.024L O_2/L/d	Continuous	0.013-0.024	99% H_2S removed. No negative effect on methane yield	(Fdz.-Polanco et al., 2009)
H_2S removal	Fluidized bed (1.7L)	Vinasse	0.7-0.9L air/L/d (Molar ratio of O_2/S is 8-10)	Continuous	0.15-0.19	Complete removal of H_2S in biogas. No aerobic respiration of organic substrate	(van der Zee et al., 2007)
H_2S removal	UASB (10.5L)	Pulp mill wastewater	3-6 mL air/L/min (0.38 mL O_2/mgS)	Continuous	0.9-1.8	Up to 30% of H_2S removal. 40-80% enhance COD removal rate.	(Zhou et al., 2007)
H_2S removal	CSTR (50L)	Primary sludge	0.14 mL O_2/sec to maintain ORP between -320 to 270mV	Semi-continuous ORP based	-	99% H_2S removal. No effects on VS and COD removal rate	(Nghiem et al., 2014a)
H_2S removal	Sludge digester (200L)	Waste Activated Sludge	0.25 L O_2 / L sludge	Continuous	0.01	98% H_2S removal from biogas.	(Díaz et al., 2011b)

H_2S removal	UASB (2.7L)	Synthetic brewery wastewater	0.5 mol O_2/S^{2-} (1L air/d)	Continuous	0.08	73% H_2S removal.	(Krayzelova et al., 2014)
H_2S removal	Sludge digester (200L)	Waste activated sludge	0.25L O_2/L feed and 1.27 L air/L feed	Continuous	0.25	99% H_2S removal.	(Díaz et al., 2010)
Control VFA accumulation and improve effluent quality	CSTR (10L)	Waste activated sludge	1.6 L /d	Continuous	0.03	Micro-aerobic reactor had 3.5 times lower VFA, 33% lower of sCOD, lower foaming and better dewaterability as compared with anaerobic reactor	(Jenicek et al., 2014)
Overcome overloading and improve reactor stability	CSTR (200L)	Waste activated sludge	4.4 NL $O_2/m^3/d$	Continuous	0.01	Overcome hydraulic overloading. Promoted growth of hydrogenotrophic methanogens.	(Ramos and Fdz-Polanco, 2013)
pH control	CSTR (3L)	Synthetic wastewater	0.1 and 1 g O_2/L.d at flowrate 0.9L O_2/min	Semi-continuous time- based	0.07 and 0.7	Help recover pH to neutral. Good for startup reactor.	(Zitomer and Shrout, 1998)
VFA production	Leach-bed reactor (1L)	Grass silage	1 L and 4 L air/min	Semi-continuous time based	0.5 and 5	Increased the VFA production by 4 times. Extensive aeration reduced VFA production	(Jagadabhi et al., 2010a)

VFA production	Batch (0.16L)	Napier grass	15 and 30 mL O_2/gVS	Single	0.09 and 1.9	Highest VFA production with 15 mL O_2/gVS and 3 days incubation time with cattle manure inoculum	(Sawatdeenarunat et al., 2017)

Note: CSTR: continuous stirred-tank reactor; UASB: up-flow anaerobic sludge blanket; sCOD: soluble chemical oxygen demand; VS: volatile solid; TS: total solid. *Calculated from reported micro-aeration intensity with assumption O_2 = 21% v/v of air.

2.3.5 Micro-aeration process control

Process control is critically important to allow precise control of micro-aeration in AD system. Besides time-based on-off control, other fine process control systems such as proportional-integral-derivative (PID) control (Liu et al., 2011) and ORP-based control (Chang et al., 2014; Khanal and Huang, 2006; Nghiem et al., 2014) are recommended. The use of ORP as a control parameter could maintain enough residual oxygen without inhibiting obligate anaerobes, since dosing of oxygen could be controlled precisely based on oxygen consumption rate of facultative bacteria in the system. Application of ORP for process monitoring and control of fermentation process for producing desired products by sensing intracellular metabolic profile was also implemented in microaerobic and anaerobic environments (Liu et al., 2013). For these reasons, ORP-based process control system appears to be the most appropriate technique for controlling micro-aeration. However, ORP is also susceptible to environmental factors such as pH, temperature, and ionic strength. Therefore, further development and application of ORP-based micro-aeration control strategy should be examined for AD system.

Selection between air and pure oxygen for dosing into the anaerobic reactor should be based on the purpose of the micro-aeration and the associated cost. Use of air is much cheaper than using pure oxygen. However, air dilutes the biogas with nitrogen, which ultimately deters the quality of biogas as an energy resource. If the sole purpose of micro-aeration is to increase the VFA production instead of biogas production, air could be used for micro-aeration. Díaz et al. (2015) suggested the application of oxygen at concentration of 95% (by volume), which was generated from pressure swing adsorption generator as a more economical substitute for air or pure oxygen.

The challenge associated with automatic process control of micro-aeration is the bottle neck of the implementation of micro-aeration at full-scale AD system. The development of an automatic micro-aeration dosing system with intrinsic control and long-term stability is required to make this technology successful.

2.4 Syntrophic relationships of microbial community under micro-aerobic condition

The foundation of AD process is the breakdown of complex organic matters in series of steps, in which products of the previous step are substrates for the following step. As a result, microorganisms interact with each other in such a way that allows the continuous substrate flow from hydrolysis to fermentation and then methane generation. This syntrophic relationship between bacteria and archaea community allows them to survive even with limited ATP production from thermodynamic marginal reactions (Stams and Plugge, 2009).

The AD process is a slow-rate process (with hydrolysis or methanogenesis as rate-limiting steps, depending on substrate type) and is highly susceptible to changes in environmental conditions including inhibitory compounds produced during AD such as H_2S, NH_3 and VFA (Chen et al., 2008). Micro-aeration overcomes these impediments by augmenting the abundance and activity of the targeted group of microorganisms in order to achieve the specific objective. For example, it stimulates the production of extracellular hydrolytic enzymes in hydrolytic bacteria thereby enhancing substrate solubilization and VFA production or promote the activity of sulfide-oxidizing bacteria for hydrogen sulfide removal.

Nevertheless, the balance and syntrophic relationship between different microbial groups have to be maintained in micro-aerobic process. In details, the micro-aeration system has to maintain the balance between anaerobic and aerobic, between OTR and OUR, between oxidation and reduction reactions, between substrates and products. In order to achieve this, synergies between sulfate-reducing bacteria and sulfide-oxidizing bacteria, hydrolytic and fermentative bacteria, methanogens and syntrophs needed to be maintained.

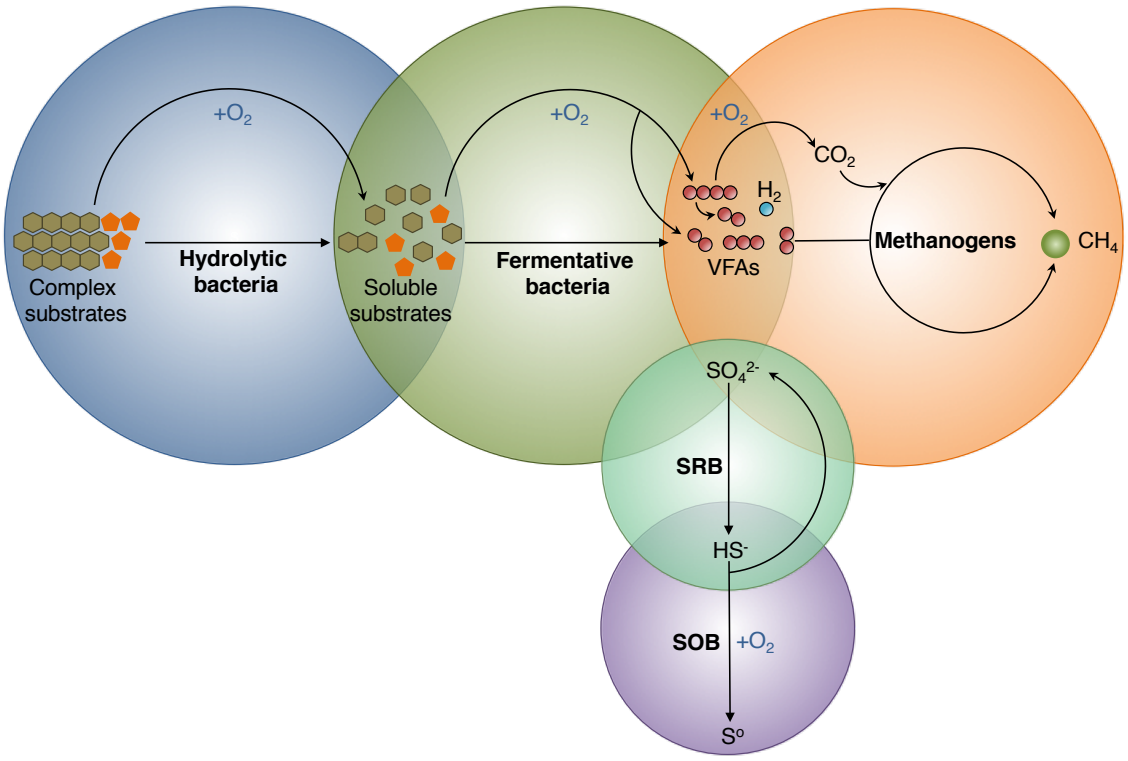

Figure 2.9 Syntrophic relationship between microbial community of AD processes and effects of micro-aeration

Table 2.6 Effect of micro-aeration on functional microbial groups

Micro-aeration purpose	Effect on microbial group (% change*)	Microbial function	Reference
Enhance hydrolysis	*Firmicutes* (+6 %)	Hydrolytic bacteria	(Fu et al., 2016)
	Clostridiales (+16%)	Hydrolytic bacteria grew on cellulosic substrate	
	Methanosarcina (+111%)	Aerotolerant methanogens	
	Methanobacterium (+100%)	Hydrogenotrophic methanogens	
Enhance hydrolysis	*Firmicutes* (+24%)	Hydrolytic bacteria grew on various substrates	(Lim et al., 2014)

VFA production	*Firmicutes* (+128%)	Hydrolytic bacteria with extracellular enzymes	(Yin et al., 2016)
Sulfide removal	*Desulfovibrio psychrotolerans* (-55% to -100%)	Sulfate-reducing bacteria	(Chang et al., 2014)
Sulfide removal	*Arcobacter mytili, Thiobacillus, Halothiobacillus, Sulfuricurvum, Acidithiobacillus* (identified)	Sulfide-oxidizing bacteria	(Díaz et al., 2011b; Ramos et al., 2013)

Note: *Changes (+: increase, -: decrease) in relative abundance (%) as compared to the same process at anaerobic condition.

2.4.1 Sulfide-oxidizing bacteria and sulfate-reducing bacteria

Sulfate-reducing bacteria (SRB), such as *Desulfobacterium, Desulfovibrio, Desulfomicrobium,* reduce sulfate to a toxic and highly corrosive hydrogen sulfide (H_2S) in biogas and dissolved sulfide (H_2S and HS^-) in aqueous phase that inhibits anaerobes especially methanogens and is corrosive to the pipe and metal parts of reactors. In addition, competition for substrates (acetate, H_2, and other intermediates) between SRB and methanogens is equally important to consider, especially when the goal is to enhance methane production (Figure 2.9). SRB with higher substrate affinity for acetate, H_2 and higher growth rate outcompete methanogens thereby resulting in low methane yield and high H_2S content in the biogas (Muyzer and Stams, 2008).

Sulfide-oxidizing bacteria (SOB) are responsible for biological oxidation of sulfide (HS^-) to elemental sulfur (S^o), sulfate (SO_4^{2-}) and thiosulfate ($S_2O_3^{2-}$) by producing Sox protein complex that catalyzes the sulfide oxidation reactions. Majority of SOB belong to phylum *Proteobacteria* with *Thiobacillus, Halothiobacillus*, *Sulfuricurvum* and *Acinetobacter* as main genus (Krayzelova et al., 2015). In microaerobic condition, dominant SOB are chemolithotrophs, and use oxygen as an electron acceptor and CO_2 or/and organic compounds as carbon and energy source. Addition of oxygen enhanced SOB activity with over 99% removal of H_2S from biogas (Jenicek et al., 2011).

Co-existence of SOB and SRB occurs in bioreactor fed with sulfate-rich substrates, where SRB reduce SO_4^{2-} to sulfide and SOB consume O_2 to oxidize sulfide into elemental sulfur (S^o) and/or SO_4^{2-} (Figure 2.9). Under microaerobic condition, the oxygen-sensitive SRB normally exist at oxygen-depleted zone and are protected from oxygen toxicity by SOB activity (Stewart and Franklin, 2008). Some species of SRB with aerotolerance such as *Desulfovibrio oxyclinae*, were also reported to grow independently under oxic condition, while other species tend to co-exist with aerobic or facultative bacteria as an oxygen-defense mechanism (Muyzer and Stams, 2008). This interaction is pivotal in controlling the micro-aeration system, since at low sulfate concentration, the activities of SRB and SOB are also low due to limited substrate availability. Hence, the injected oxygen is not completely consumed by SOB thereby resulting in the inhibition of strict anaerobic SRB and methanogens (Khanal and Huang, 2003).

Air or oxygen is injected into reactor headspace and the holding time of biogas in headspace is the main factor governing the H_2S removal efficiency (Krayzelova et al., 2015). Several studies on micro-aeration of liquid phase of the anaerobic reactor were able to remove both H_2S in biogas and dissolved sulfide in liquid. (Díaz et al., 2011b; Khanal and Huang, 2006). Micro-aeration rate for sulfide removal can be calculated using molar ratio of O_2/S^{2-}. Stoichiometrically, 0.5 mole O_2 per mole S^{2-} is required for converting H_2S into S^o or SO_4^{2-}. The micro-aeration rate, however, is affected by various factors, such as sulfide concentration, soluble substrate concentration, and pH among others. Therefore, a wide range of O_2/S^{2-} molar ratios (i.e. 0.15 to 2.0) have been reported in the literature (Krayzelova et al., 2015).

Although micro-aeration was able to remove over 99% of sulfide, reduced methane yield due to aerobic oxidation of substrate resulting from excess aeration and pipe clogging associated with sulfur element deposits, were reported as the major challenges of biological desulfurization using micro-aeration (Krayzelova et al., 2015). The application of precise automatic process control for micro-aeration, such as PID control (Ramos et al., 2014), and ORP-based control with supervisory control and data acquisition (SCADA) system (Nghiem et al., 2014) can minimize the issue with excessive substrates oxidation. Additionally, the micro-aeration membrane dosing system, which allows

elemental sulfur to precipitate on the removable membrane surface, can help to remove the deposited sulfur particles and overcome the pipe-clogging problem (Camiloti et al., 2014; Krayzelova et al., 2015).

2.4.2 Hydrolytic and fermentative bacteria

Taxonomic analysis of 21 full scale AD plants showed that *Bacteria* domain consisted of more than 80% of total DNA sequences (Sundberg et al., 2013). Among bacteria, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* were the dominant bacterial phyla. However, this microbial structure in AD processes varied depending on inoculum types (Yin et al., 2016), reactor configurations (Lim et al., 2013), stages of operation (i.e. startup or stable phase) (Goux et al., 2016), and substrate types (Hanreich et al., 2013). Metaproteomic study found out that *Fimicutes* and *Bacteroidetes* phylum are responsible for the hydrolysis of hemicellulose, cellulose and other polysaccharides, while *Proteobacteria* are mainly glucose and VFA utilizing bacteria (Ariesyady et al., 2007; Hanreich et al., 2013)..

Micro-aeration facilitates the establishment of more diverse hydrolytic and fermentative bacterial communities with higher activity in AD systems (Fu et al., 2016; Lim et al., 2014). Enhancement of diversity and activity of these facultative bacteria is the foundation for controlling the VFA concentration (either enhance or reduce), which promotes the overall stability of the AD process. Compared to strict AD system, microaerated AD system consisted of a higher proportion of *Firmicutes* phylum that is associated with higher substrate hydrolysis rate (Table 2.6). For example, *Firmicutes* population shifted from 58% to 72% after micro-aeration treatment (Lim et al., 2014). Upon micro-aeration, augmentation of bacterial activity in phylum *Firmicutes* (especially in *Clostridia* and *Bacilli* class) resulted in a 3-folds increase in acetic and butyric acids concentration, which ultimately resulted in higher methane yield (Xu et al., 2014).

Under microaerobic condition, facultative anaerobes were more likely to switch their intracellular metabolic pathway from anaerobic fermentation to aerobic respiration, which is much more thermodynamically (Table 2.2) and kinetically favorable (Table 2.3). Central regulators such as fumarate nitrate reduction (FNR) and anoxic redox control

(AcrA/ArcB) upregulate the expression of genes involved in anaerobic metabolism (Forster and Gescher, 2014). Free O_2 under microaerobic condition can inactivate these central regulators, leading to the expression of genes related to aerobic metabolism. For example, deletion of *arcA* and *fnr* genes encoding these two regulators caused overexpression of genes related to TCA cycle such as *sucC* (succinyle-CoA synthase) and *sdhC* (succinate dehydrogenase) as compared to wild type (Shalel-Levanon et al., 2005).

Hydrolysis is reported to be the rate-limiting step in AD of high solid organic substrates such as municipal solid wastes and lignocellulosic biomass (Shrestha et al., 2017). Micro-aeration is a promising environment friendly biological pre-treatment technique to enhance methane yield from such recalcitrant substrates. The increase in extracellular hydrolytic enzymes (e.g. amylase, protease and cellulase) production from a more abundant and diverse hydrolytic bacterial communities under microaerobic condition enhances the hydrolysis of carbohydrates, proteins, and other complex organic substrates (Lim et al., 2014; Zhu et al., 2009). For instance, Johansen and Bakke (2006) reported up to 60% enhancement in hydrolysis after 4 days of microaerobic pretreatment. Similar trends were also found in recent studies with various substrates such as municipal solid waste (Nguyen et al., 2007), vegetable waste (Zhu et al., 2009), food waste (Xu et al., 2014), grass silage (Jagadabhi et al., 2010b), sugarcane bagasse (Fu et al., 2015), co-digestion of food waste and domestic wastewater (Lim and Wang, 2013), and Napier grass (Sawatdeenarunat et al., 2017).

Augmentation in hydrolytic activity results in higher soluble substrate availability for fermentative bacteria to produce VFA and other fermentative products, which could serve as potential substrates for producing high value alcohol-based fuels, biopolymer, and electricity using microbial fuel cell (Botheju and Bakke, 2011; Jagadabhi et al., 2010b; Sawatdeenarunat et al., 2016). As a result, microaerobic AD process could be used to produce VFA as the final products instead of biogas; and since the strict anaerobic methanogens are excluded from the process, there is much greater flexibility to control micro-aeration in the acid-producing digester. Both the yield and composition of VFA strongly depend on the substrate types and operating conditions including temperature, pH, OLRs, hydraulic retention time, and solids retention time (Sawatdeenarunat et al.,

2017; Surendra et al., 2015). Thus, to optimize AD process to maximize VFA yield, the above stated operating conditions should be maintained at the optimum range. During acidogenesis, the optimum range of ORP to facilitate VFA production is reported to be between -100 and -200 mV (Yin et al., 2016). At ORP higher than -100 mV, propionic acid was reported to be the dominant VFA (Wang et al., 2006). The shift in fermentation pathway such as increase in butyric acid production from conventional acetic acid producing pathway was achieved under microaerobic condition (Lim et al., 2014). These results can be applied to control ORP level using micro-aeration to enhance the yield of specific VFA. However, further molecular and multi-omic analysis needs to be conducted to better understand the shift in metabolic pathway under microaerobic condition.

Micro-aeration promotes the diversity, growth and activities of rapid-growing facultative heterotrophs which would prevent the accumulation of VFA in AD processes operating at high OLRs (Guo et al., 2015). For example, under microaerobic condition, propionic, butyric and valeric, and lactic acid were converted into acetic acids more efficiently (Lim and Wang, 2013). In addition, hydrogenotrophic methanogenic pathway was promoted under microaerobic condition that maintained low hydrogen partial pressure thereby maintaining stable pH of the reactor while efficiently producing methane yield (Ramos and Fdz-Polanco, 2013).

Lower effluent COD, better sludge dewaterability and significantly lower foaming problem of an AD process were reported during long-term operation under microaerobic condition (Jenicek et al., 2014). Nghiem et al. (2014), however, observed no significant differences in pH, VS removal, alkalinity, and methane production between anaerobic and microaerobic conditions. This could be due to rapid oxygen consumption by SOB for sulfide removal in which H_2S in biogas decreased from over 6,000 ppm to 30 ppm. Thus, the fate of oxygen in microaerobic system strongly depends on synergetic activities and substrate (oxygen) competition among different microbial groups.

In conclusion, hydrolytic and fermentative bacteria, as the most abundant microbial group in microaerobic condition, improve the AD process in multiple ways. The enhancement in diversity and activity of these bacteria with the availability of limited oxygen is the key for the operation of an efficient AD system. With the controlled dosing

of appropriate amount of oxygen, improvement in VFA production and methane yields, and overall system stability could be achieved.

2.4.3 Methanogens and syntrophs

Even though *Archaea* domain usually consists of just <4% of microbial population, metagenomic study has shown that around 30% of protein released in AD process belongs to this domain (Hanreich et al., 2013). Acetoclastic *Methanosaeta, Methanosarcina* and hydrogenotrophic *Methanosarcina, Methanoculleus, Methanobacteriales,* and *Methanospirillum* are the main methane-producing archaea with the typical higher proportion of acetoclastic methanogens (Lim et al., 2013; Tang et al., 2004). However, in AD processes with high concentrations of VFA, ammonia and high temperature (55^{o}C, thermophilic AD), hydrogenotrophic methanogenesis is the dominant pathways (Hattorii, 2008). This could be due to hydrogenotrophic methanogens (HM) and syntrophic acetate-oxidizing bacteria (SAOB) are more resilient to inhibitors than the acetoclastic *Methanosaeta* (Demirel and Scherer, 2008; Wang et al., 2015).

Enhancement in hydrolysis and acidogenesis under microaerobic condition generates more substrate for methanogens that resulted in higher specific methanogenic activity and ultimate methane yield under microaerobic AD process (Fu et al., 2016; Lim and Wang, 2013). In addition, as discussed previously, the removal of sulfide and stabilization of pH in microaerobic process also create an optimum condition for the growth of methanogens. Micro-aeration also directly affects the methane production step by modifying the dominant methanogenic pathway. With the ability to use both acetate and hydrogen to produce methane, along with aerotolerance, *Methanosarcina* was found to be the dominant archaea in microaerobic system (Fu et al., 2016). The shift of dominant archaeal genus from *Methanosarcina* to *Methanoculleus* and from acetotrophic to hydrogenotrophic pathway following micro-aeration was reported in several studies (Ramos and Fdz-Polanco, 2013; Tang et al., 2004). The shift between acetoclastic and hydrogenotrophic pathway is highly dependent on the activity of SAOB (converting acetate to H_2 and CO_2) and homoacetogenic bacteria (reducing CO_2 to acetate). *Thermacetogenium phaeum, Syntrophaceticus schinkii, Tepidanaerobacter acetatoxydans* and *Clostridium ultunense* are the common SAOB (Ariesyady et al.,

2007). Dominance of SAOB and hydrogenotrophic methanogens was found in co-digestion of diverse substrates in full-scale AD systems in contrast to the acetoclastic methanogens for AD process fed with sewage sludge (Sundberg et al., 2013).

Under microaerobic condition, facultative heterotroph could join the syntrophic relationship between methanogens and syntrophs as previously discussed. The traditional syntrophic interaction between SAOB and hydrogenotrophic methanogens could be replaced by the synergetic interaction between facultative bacteria (producing H_2, CO_2) and hydrogenotrophic methanogens (consuming H_2, CO_2). The later combination is highly energetic (Table 2.2) and could promote the stability of AD processes operating at high OLRs, if micro-aeration is meticulously controlled.

Therefore, changes in VFA, H_2 and CO_2 concentration of AD processes operating under microaerobic condition can affect the archaeal communities and therefore the dominant methanogenic pathway. This interesting concept needs to be examined to better understand the dynamic interaction between bacterial and archaeal communities using novel multi-omics approaches.

Chapter 3

Materials and Methods

3.1 Overall research framework

The overall goal of this study is to develop and validate the performance of an ORP-based micro-aeration system for controlling VFA accumulation and maintaining stability of AD process at high OLRs. The specific objectives are to: (1) identify key monitoring parameters to estimate reactor performance; (2) develop an ORP-based micro-aeration process control system for the AD process; and (3) evaluate the effect of micro-aeration on microbial community of the AD process. Figure 3.1 shows the overall research framework of this study.

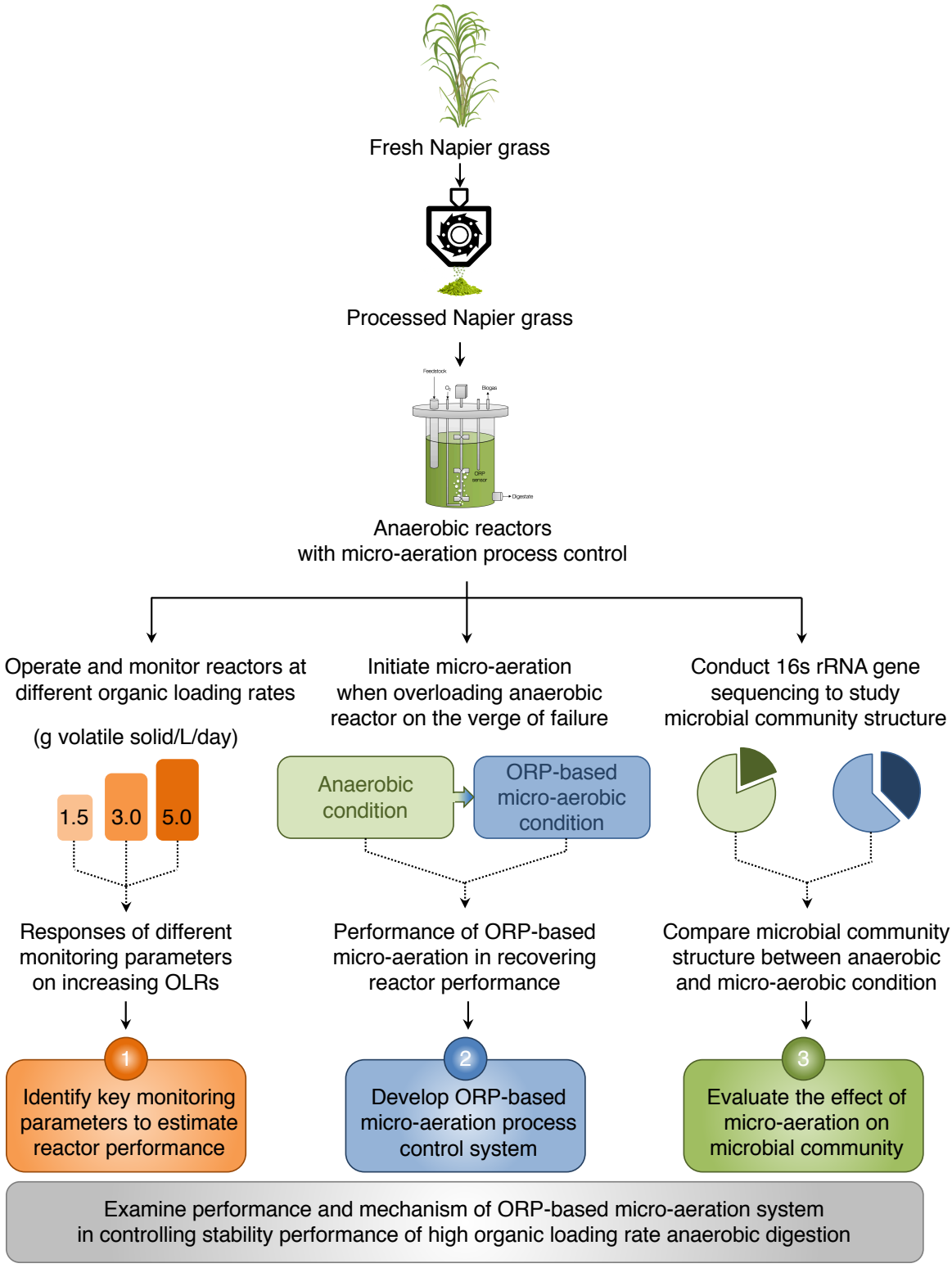

Figure 3.1 Overall research methodology according to objectives

3.2 Feedstock and inoculum

Napier grass (*Pennisetum purpureum*, 5 months old) was harvested from the Waimanalo Research Station (Waimanalo, HI, USA). The freshly harvested grass was passed through a shredder (Vincent Corporation, Tampa, FL, USA) and was air-dried until moisture content reached <15% for stable storage at room temperature. The dried biomass was then passed through a cutting mill (Retch SM2000, Haan, Germany) with a screen size of 2 mm. The processed biomass was stored in vacuum bags at room temperature and was used as a sole feedstock for the entire research period. The processed biomass was characterized for total solids (TS), volatile solids (VS), carbon-to-nitrogen (C/N) ratio, and fiber composition (Table 3.1).

Cattle manure-derived inoculum, which was maintained in a 20-L inoculum reactor at mesophilic condition (35 ± 2^{o}C), was used to start-up the reactors. The inoculum reactor was previously fed with fresh cattle manure and the inoculum was harvested after 40 days of digestion by passing through a #8 sieve (ASTM 2.36 mm, Thermo Fisher Scientific Inc., USA) to remove residual fibers in the inoculum. The prepared inoculum was characterized for TS, VS, C/N ratio, and total VFA to total alkalinity (VFA/ALK) ratio (Table 3.1). The inoculum was stored anaerobically at 4^{o}C in walk-in refrigerator until further use.

Table 3.1 Characteristics of inoculum and feedstock for the AD reactor

	Feedstock	**Inoculum**
TS (%)	88.5 ± 0.1	3.7 ± 0.0
VS (% TS)	89.1 ±0.2	73.1 ± 0.1
Hemicellulose (% TS)	26.6 ± 0.0	NA
Cellulose (% TS)	42.1 ± 0.7	NA
Acid detergent lignin (% TS)	5.3 ± 0.7	NA
C/N ratio	56.7 ± 1.7	3.1 ± 0.1
VFA/ALK	NA	0.1 ± 0.0

Note: Data shown as mean ± standard deviation of triplicated samples. NA: Not available

3.3 Reactor set-up and operation

A 3.5-L continuous stirred tank reactors (CSTRs) with a working volume of 2 L were fabricated using acrylic (Figure 3.2). Mesophilic temperature (35 ± 2°C) was maintained using a rubber heating jacket with temperature controller (Power density: 1.25 W/inch^2, Brisk Heat, OH, USA). To start up, the reactor was loaded with 1.5 L of inoculum and purged with N_2 gas in 15 min to create anaerobic condition. Napier grass was gradually fed into the reactor at OLR 0.5 g VS/L/day for 20 days (until the reactor's working volume reached 2 L). The reactor was then operated at OLR of 1.5, 3.0, and 5.0 g VS/L/day. The OLR was increased gradually at incremental rate of 0.5 g VS/L/day (Figure B2 to B4). The reactor was operated in the semi-continuous mode by daily withdrawing digestate and feeding mixture of Napier grass and distilled water to maintain hydraulic retention time (HRT) of 20 days. Reactor was operated at anaerobic condition and micro-aeration was initiated when the reactor showed the sign of instability, especially VFA accumulation. Reactor performance was evaluated based on methane yield, pH, ORP, VFA/ALK ratio, total VFA, individual VFA, and VS removal. The whole set of experiment was performed in triplicate for statistical analysis.

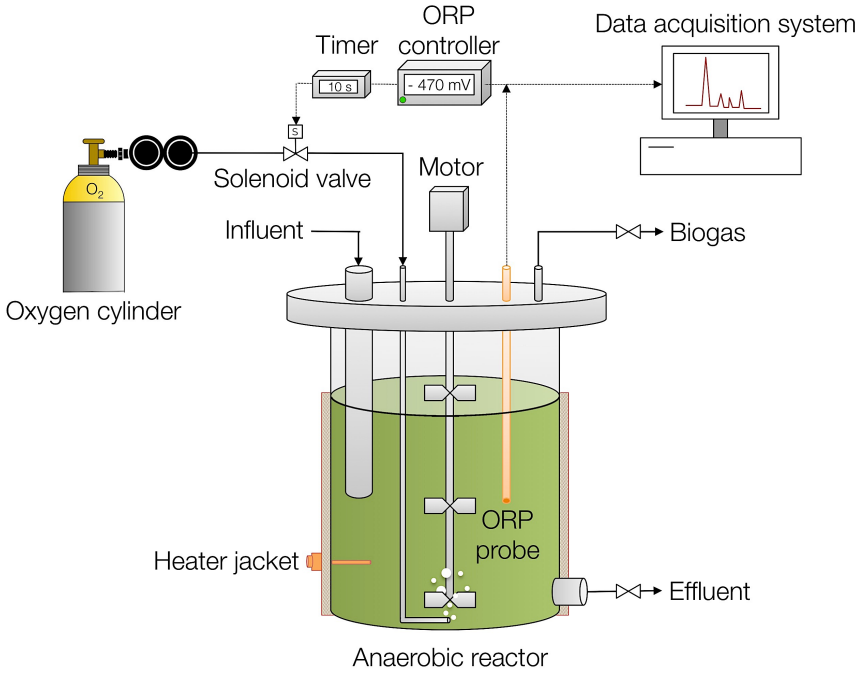

Figure 3.2 Schematic diagram of reactor set-up with ORP-based micro-aeration system

3.4 ORP-controlled micro-aeration system

The reactor as depicted in Figure 3.2 are equipped with thermocouple (TTSS-116U-12, Omega Engineering, CT, USA) and oxidation-reduction potential (ORP) probe with Ag/AgCl reference electrode (EW-27003-40, Cole-Parmer, IL, USA) to monitor temperature and ORP, respectively using data loggers (Dataq, OH, USA). The ORP probe was connected to an ORP controller (EW-56700-00, Cole-Parmer, IL, USA) to control the micro-aeration system. The flow rate of oxygen from the compressor tank (OX R200, Airgas, HI, USA) was finely adjusted at 10 mL/min (to meet adequate pressure for oxygen injection) using the two-staged low-pressure regulator and needle valve. A digital timer was used to automatically monitor the total micro-aeration time in seconds. Daily oxygen dosing volume (mL/day) was then calculated by multiplying the oxygen flow rate with daily micro-aeration time.

ORP or redox potential, which is highly sensitive to the dissolved oxygen (DO) concentration, was selected as the control parameter for micro-aeration in this research. A linear correlation between ORP and logarithm of DO concentration was observed as shown in Figure A.3, indicating that even a small amount of DO (< 0.1 mg/L) could be detected by the ORP probe. According to the correlation, DO of 0.1 mg/L is equivalent with ORP of -96 mV; and a 25 mV increase in ORP is equivalent to a DO increase of 0.02×10^{-6} mg/L or 1.25×10^{-6} μM O_2 (Figure A.3).

When oxygen is supplied to an anaerobic process, it creates a more oxidative environment, resulted in a rise in the ORP to a more positive value. The redox potential difference between anaerobic and micro-aerobic conditions was reported to be at +25mV (Khanal and Huang, 2006; Krayzelova et al., 2014), +35mV (Jenicek et al., 2011) and +50 to +100 mV (Khanal and Huang, 2003). In this research, micro-aerobic conditions were maintained via ORP-controlled micro-aeration at a target ORP of +25 mV above the baseline ORP of an anaerobic bioreactor, following the control scheme previously described (Khanal and Huang, 2003). When the ORP of the reactor reached 10 mV below the target value, the ORP controller triggered the solenoid valve (DVP-2DC1D, Automation Direct, GA, USA) to inject oxygen into the bottom of the reactor via a 2 mm

inner diameter stainless steel tube. The injected oxygen promptly spiked up the ORP to approximately 10 mV above the target value, which then prompted the solenoid valve to close thereby stopping the micro-aeration. The ORP then gradually reduced until it reached below the set point and again that started the next cycle of micro-aeration. Hence, a uniform ORP profile oscillating between 10 mV below and above the target ORP was obtained.

3.5 Chemical analyses

The pH was measured daily using a pH meter (Accumet AB15, Fisher, OH, USA). ORP was measured on-line (at sampling rate of 10 mins/sample) using an ORP probe with Pt electrode and Ag/AgCl reference electrode filled with 3M KCl (EW-27003-40, Cole-Parmer, IL, USA), calibrating with Zobell standard solution (Fisher, OH, USA). All ORP values in this study were reported in mV with Ag/AgCl as a reference electrode (E_{Ag}), otherwise specified. The standard redox potential value (E_h) with standard hydrogen electrode at 35^{o}C can be calculated according to the manufacturer's guideline using the following expression: $E_h = E_{Ag} - 200$ mV. ORP value with adjustment to pH 7 ($E_{h\ pH\ 7}$) could be done using correlation in Appendix A.

Daily biogas production was collected in a 10-L Tedlar gas bag (CEL Scientific Corporation, CA, USA) and quantified using a milli-gas counter (Ritter US LLC, NY, USA). Gas volumes were normalized to standard temperature (273 K) and pressure (1 atm) and reported as NmL. Biogas composition (i.e., CH_4, CO_2, N_2, H_2 and O_2) was determined using gas chromatography equipped with thermal conductivity detector (GC-TCD) at 80^{o}C (GC2014, Shimadzu, Japan) using a packed column (80/100 Hayesep D column, 2 m x 2.1 mm ID, Supelco, PA, USA). The column temperature was programmed at 80^{o}C and held for 40 min. Daily methane yield was calculated and reported as NmL CH_4/g VS added.

Digestate samples from AD reactor were centrifuged at 10,000 rpm in 10 min (Eppendorf 5810R, Germany) before filtered through GF/C glass microfiber filter paper (Whatman 1822-047, UK). The filtered samples were then used for total VFA, total alkalinity, and individual VFA analysis.

Total VFA to total alkalinity (VFA/ALK) ratio was analyzed every two days using an autotitrator (TitraLab AT1000, Hach, Germany) with FOS/TAC software (HACH, Germany) based on the method of (Kafle et al., 2012). The VFA/ALK ratio was reported as mass ratio of acetic acid (HAc) equivalent over $CaCO_3$ equivalent. The total VFA values from the autotitrator were reported as g/L as HAc and were not substantially different ($p > 0.05$) with the values obtained by adding the concentrations obtained for individual VFA.

Individual VFA (acetic, propionic, butyric, iso-butyric, valeric, and iso-valeric acids) were determined every two days using gas chromatography equipped with flame-ionization detector (GC-FID) (GC2014, Shimadzu, Japan) using a capillary column (ZB-Wax Plus column, 30 m x 0.25 mm x 0.25 μm, Phenomenex, CA, USA). Helium was used as the carrier gas at a flow rate of 2 mL/min. The temperature was programmed to increase from 50^{o}C to 180^{o}C at a rate of 20^{o}C/min, and held for 10 min, the split ratio was 20, the purge flow was 1 mL/min, and injector and detector temperatures were 250^{o}C each. The samples for individual VFA analysis were first reduced to pH < 2 with 17% H_3PO_4 and then filtered through 2μm nylon filter tip to convert VFA to unionized from and remove residues, respectively. Dilution of VFA concentrations from added H_3PO_4 was taken into account in final calculation. TS and VS were analyzed following Standard Method (APHA-AWWA-WEF, 2005). Fiber compositions (neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL)) were analyzed using a cell wall fractionation method (Faithfull, 2002) using the Fiber Analyzer (ANKOM 200, ANKOM, NY, USA) with equations: hemicellulose = ADF-NDF; cellulose = ADL-ADF, lignin = ADL; extractive = 1-NDF-Ash; ash=1-VS.

For the carbon mass balance analysis, the carbon contents of influent (feedstock) and effluent (digestate) samples were achieved from total carbon content analysis (g C/g VS) at Agricultural Diagnostic Service Center, University of Hawai'i at Manoa; data of CH_4 and CO_2 were converted from gas production at standard temperature and pressure (273 K and 1 atm) using ideal gas law equation PV=nRT; data of "Biomass & Intermediates" representing unmeasured components were calculated from influent carbon (4.88 g C/day at OLR 5.0 g VS/L/day) minus carbon content from other components.

3.6 Microbial analyses

Biomass samples from anaerobic and micro-aerobic conditions were collected from the reactor at OLR of 5 g VS/L/day. DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Canada) as per the manufacturer's instructions.

The bacterial and methanogenic communities were investigated by targeting the 16S rRNA gene using 515F/806R primers and the methyl coenzyme-M reductase (*mcrA*) gene using mcrA3F/mcrA3R primers, respectively (Caporaso et al., 2012; Luton et al., 2002). Sequencing was performed using the Illumina HiSeq2000 platform and sequence reads were processed using mothur (version 1.35.0) (Schloss et al., 2009). Chimeras were removed using UCHIME (Edgar et al., 2011). Bacterial 16S rRNA gene fragments were aligned against the SILVA database (release 123) (Pruesse et al., 2007) and *mcrA* gene fragments against a subset of FunGene database (Fish et al., 2013), resulting in taxonomic labels for each aligned fragment.

3.7 Statistical analyses

Statistical significance between 2 conditions was determined by analysis of variance (ANOVA). The post-hoc Tukey's Honest Significant Difference (HSD) test was used to compare differences among 3 or more conditions. All statistical analyses were done on JMP software (JMP Pro Version 12.0, SAS Institute Inc., NC, USA) with p-value of 0.05.

Chapter 4

Results and Discussion

4.1 Performance of anaerobic reactor at increasing organic loading rates

The anaerobic reactor was operated at incremental OLRs of 1.5, 3, and 5 g VS/L/day in triplicate experimental sets to record the reactor performance at stable operating condition. The stable operating condition is defined when there is no significant difference (p>0.05) in each parameter during 5 consecutive days. The effect of increasing OLRs on the performance of the anaerobic reactor in triplicate experiments is shown in Figure 4.1 and Table 4.1.

4.1.1 Methane yield

Stable operation was achieved at OLRs of 1.5 and 3 g VS/L/day with an average methane yield of 104.51 ± 5.87 and 101.86 ± 5.87 NmL/g VS added, respectively. As compared to the biochemical methane potential (BMP) of 124.68 ± 5.94 NmL/g VS added (Figure B.1), the methane production of the semi-continuous reactors at these two OLRs are relatively high (corresponded to 82-83% of BMP), indicating the good performance of the reactor. Methane production recorded in this study is in great agreement with AD reactors fed with Napier grass reported in literature (Sawatdeenarunat et al., 2018; Surendra and Khanal, 2014). The methane production, however, significantly reduced to 80.95 NmL/g VS added (equivalent to 65% of BMP) at OLR of 5 g VS/L/day. Thus, the methane yield reduced by 20.5% as the OLR increased from 3 to 5 g VS/L/day, indicating the disturbances caused by overloading.

The ratio of CH_4/CO_2 composition in biogas followed the same trend of methane yield, where reactors operated at OLR of 5 g VS/L/day has significantly lower CH_4/CO_2 ratio than that of the reactor during lower loading rates (1.5 and 3 g VS/L/day) (p<0.05). The CH_4 composition in biogas at OLR 5 g VS/L/day dropped down as low as 37.5% v/v in biogas from the stable level of 45-50% at OLR 1.5 and 3 g VS/L/day. As the main product of AD processes, volume of methane production directly indicates performance of the digesters. However, methane yield, methane content, or CH_4/CO_2 ratio have

lagging response over reactor inhibition (organic overloading in this case) and not suitable as early warning indicators for process instability. The reduction in methane composition and methane yield at high OLR indicates inhibition of methanogens, likely due to the accumulation of VFA and drop in pH, which could be early detected before severe performance deterioration (Figure 4.1).

4.1.2 pH

pH of the reactors significantly decreased as the OLRs increased from 1.5 to 3 and to 5 g VS/L/day ($p<0.05$). Since the optimum pH range of a mixed-culture AD system is between 6.8 and 7.4 (Table 2.1), the reduction of pH to 6.73 ± 0.02 at OLR 5 g VS/L/day adversely impact the balance in activity of the microbial consortia, especially methanogens. This was the putative cause of reduction in CH_4 content in biogas and methane yield at this high OLR as previously discussed. The responsiveness of pH to incremental OLRs, even before the significant reduction of methane yield make this simple yet robust parameter ideal for early inhibition detection and monitoring reactor performance. It is worth to note that throughout this stable operating condition of 3 OLRs, no pH adjustment was made, contributing to the responsiveness of pH to increasing OLRs.

4.1.3 Total VFA

Similar to pH, the total VFA concentration significantly increased corresponding to the incremental OLRs ($p<0.05$). The accumulation of VFA in the reactors to 3.04 ± 0.14 g/L as acetate at OLR 5 g VS/L/day could explain the drop in pH below the optimum range at this condition. Data of total VFA from autotitrator was not different ($p>0.05$) than that from GC-FID analysis. This makes the application of this budget titration method for total VFA analysis ideal for monitoring and controlling small-scale AD plants especially in developing countries as pointed out by other studies (Feitkenhauer et al., 2002; Lahav and Morgan, 2004).

The accumulation of total VFA in an AD system causes by the kinetic and energetic imbalance between VFA fermenters (hydrolytic/fermentative bacteria), VFA degraders

(syntrophic acetogenic bacteria) and methanogens (Table 2.2 and Table 2.3). Accumulation of VFA was found to effectively predict process imbalance and potential failure of AD reactors (Ahring et al., 1995; Kleyböcker et al., 2012; Pind et al., 2003). These researches also reported that the trend of total VFA concentration (accumulation) is more important to considered than their absolute concentrations, which could greatly vary depending on operational conditions (such as temperature, substrate types, reactor configuration, buffering capacity, or inhibitors concentration). Also, the VFA degradation pattern is equally important to monitor and control process performance, especially for reactors subjected to disturbances (Pind et al., 2003). As a result, pattern of VFA accumulation and degradation was applied as main monitoring parameter to evaluate reactors performances.

4.1.4. VFA/ALK ratio

The VFA/ALK ratio followed the same trend with total VFA concentration regardless of slight increase in total alkalinity at higher OLRs. The VFA/ALK ratio of reactors in this study was much higher than recommendation range of less than 0.2 (Table 2.1). At OLR of 3 and 5 g VS/L/day, the VFA/ALK ratio raised up to 0.55 and 1.07 ± 0.03, respectively. This ability of the AD system to operate at this high VFA/ALK ratio without failing could be due to the slow digestibility of recalcitrant Napier grass as sole substrate. Sugars were slowly released from the hydrolysis of hemicellulose and cellulose that allow VFA to be gradually fermented. As a result, the microbial community in the reactor could adapt to the high VFA environment and still producing methane at such high VFA/ALK ratio (Pind et al., 2003). Stable methane production at VFA/ALK ratio greater than 1 was also found in solid-state anaerobic co-digestion (Brown and Li, 2013; Wang et al., 2012). High solid content of reactors (up to 6.9% TS at OLR 5 g VS/L/day) that limit the mass transfer and could be another reason that allow microbes to operate at such high VFA concentration and VFA/ALK ratio.

4.1.5. Individual VFA

The VFA composition was mainly acetate and propionate at all 3 OLRs. At low OLR of 1.5 g VS/L/day, acetate was the main VFA component (53.9% of total VFA) followed by

propionate (44.1% of total VFA). However, propionate became the most dominant VFA (50 to 59% of total VFA) at higher OLRs of 3 and 5 g VS/L/day with concentration of 0.66 ± 0.12 and 1.7 ± 0.12 g/L, respectively. The accumulation of propionate was well reported as early warning indicator for reactor inhibition causing by organic overloading (Boe et al., 2010; Hansson et al., 2002; Nielsen et al., 2007; Ward et al., 2008). The degradation of propionate after unstable operating period was also found to be slowest comparing to other VFA, making it an ideal disturbance indicator (Pind et al., 2003). Additionally, the propionate to acetate ratio of greater than 1.4, originally proposed by (Hill et al., 1987) was also widely applied to indicate inhibition of AD reactors in recent researches (Goux et al., 2015; Wagner et al., 2014; Wang et al., 2012). In this research, the propionate to acetate ratio was at 3.2 at both OLR 3 and 5 g VS/L/day, indicating severe inhibition of syntrophic propionate-degrading bacteria.

Acetate concentration in reactors at OLR 5 g VS/L/day was significantly higher than that at 2 lower OLRs. Thus, acetate accumulation is a symptom of reactor overloading that inhibits activity of methanogens (main acetate degraders). Raise in concentration of acetic acid together with drop in pH and reduction in methane yield or methane content acted as parameters indicating severe process instability from organic overloading and signal the beginning of failure period at OLR of 5 g VS/L/day.

4.1.6. VS removal

The VS removal of the reactor reduced from 60.7% to 51.3% and then to 37% as OLRs increased from 1.5 to 3 and to 5 g VS/L/day (Table 4.1). The increase of OLR affected the TS content of feed mixture and therefore increase the solid content in the reactor from 2.65 to 6.88% TS at OLR 1.5 and 5 g VS/L/day, respectively. High solid content in the reactors at higher OLRs hindered the necessary contact between extracellular hydrolytic enzymes with cellulose and hemicellulose polymeric fibers (Shrestha et al., 2017). The deficiency in hydrolysis rate expressed in the reduction in VS removal percentage at higher OLRs. Even though the VS removal rate reacted proportionally with the increase in OLR, the analysis was tedious and results were only received after minimum 2 days of analysis. These drawbacks make this monitoring parameter unsuitable for early warning inhibition of the reactor.

4.1.7. ORP

The average ORP (E_{Ag}) of the bioreactor reduced from -473 ± 4 mV at OLR 1.5 to stable level of -501 and -503 ± 4 mV at higher OLRs of 3 and 5 g VS/L/day, respectively. Converting to equivalent ORP values with SHE at pH 7 ($E_{h\ pH\ 7}$), the ORP of anaerobic reactors ranged from -274 to -305 ± 4 mV, which is in agreement with previous researches (Figure 2.4 and Table 4.3). Details about factors affecting ORP and conversions of ORP between different reference electrodes are available in Appendix A.

4.1.8. Other parameters

The total ammonia nitrogen (TAN) of reactors at all time were < 500 mg/L from periodical analyses. Due to the low total nitrogen content (0.77 ± 0.27 % TS) of Napier grass, the TAN in the reactor was stable and under the inhibition limit of 1,000 mg/L (Table 2.1) at all time. In addition, the hemicellulose and cellulose content in digested fibers were increased but not significantly different (p>0.05) at different OLRs (Table 4.1). This could be explained by the homogenous mixing of digested fibers and fresh substrate in the reactors, making this parameter insensitive to the incremental OLRs. However, the decrease in substrate degradation at increasing OLRs was supported by the decreasing trend in VS removal rate, suggesting that at higher OLRs, the hydrolysis and organic degradation performance of the reactors were slowly deteriorated.

4.2 Key monitoring parameters to estimate reactor performances

Evaluating the response of different monitoring parameters to increases in OLRs, four parameters: pH, VFA/ALK, total VFA, and propionate were found to be most sensitive to the changes in OLR and serve as early warning indicator of reactor instability. Other parameters (methane yield, CH_4/CO_2 ratio, total alkalinity, acetate) were found to have lagging response to the organic overloading and only responsive when reactors experienced instability at OLR 5 g VS/L/day.

In conclusion, pH, VFA/ALK ratio, total VFA, and propionate were selected as key monitoring parameter to estimate reactor performance. Methane yield and acetate were useful to monitor methanogens inhibition especially during unstable period. Hence, the

performance of the ORP-based micro-aeration system on recovering process stability at high OLRs of 5 g VS/L/day were evaluated based on these key parameters.

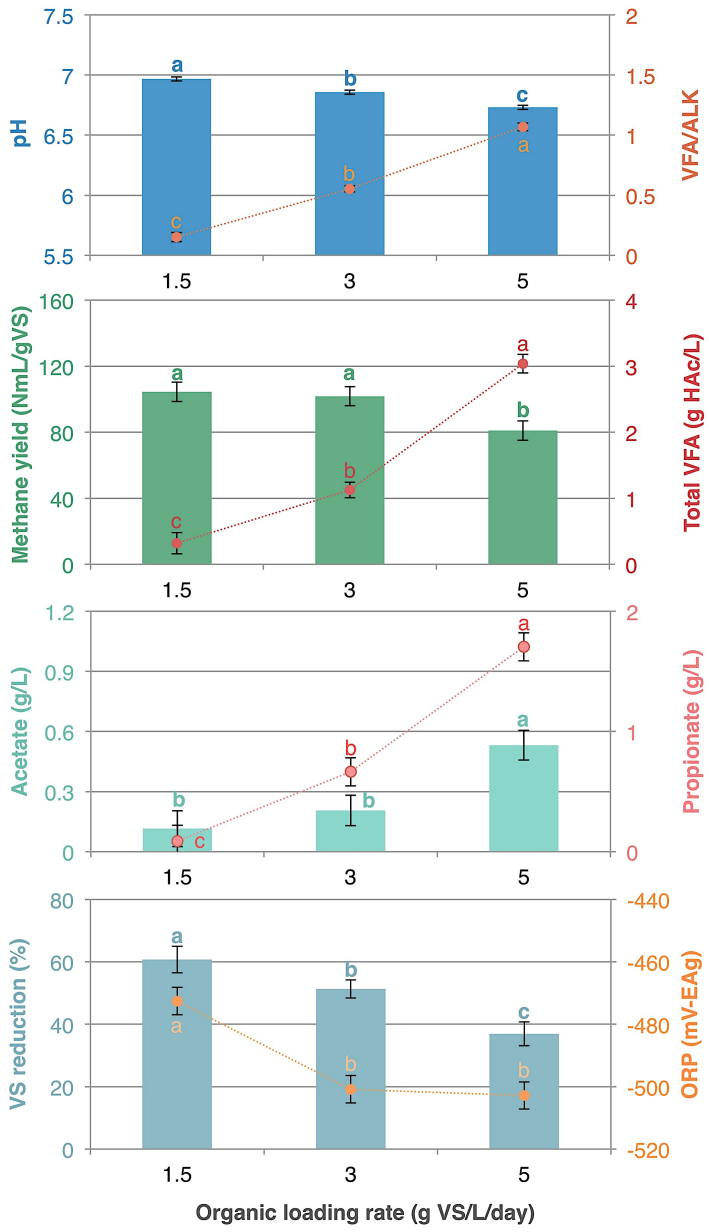

Figure 4.1 Reactors performance at increasing organic loading rates.

Note: Data shown as mean value with error bar represents standard error of triplicated experiments with 5 measurements for each parameter at stable operating condition (n=15 for each condition). Data connected by different letters are significantly different from post-hoc Tukey's test (p-value < 0.05). Left axis reads data in column format and right axis reads data in marked line format. Detail results are presented in Table 4.1.

Table 4.1 Reactors performance at increasing organic loading rates

	Organic loading rates (g VS/L/day)								
	1.5			3			5		
	Mean	SE	L	Mean	SE	L	Mean	SE	L
pH	6.97	0.02	a	6.86	0.02	b	6.73	0.02	c
ORP (mV-E_{Ag})	-473	4	a	-501	4	b	-503	4	b
ORP (mV-$E_{h\ pH\ 7}$)	-274	4	a	-303	4	b	-305	4	b
Methane yield (NmL/gVS added)	104.51	5.87	a	101.86	5.87	a	80.95	5.87	b
CH_4/CO_2 ratio	1.27	0.03	a	1.22	0.03	a	1.03	0.03	b
Total VFA (g /L as HAc)	0.32	0.16	c	1.13	0.12	b	3.04	0.14	a
Total alkalinity (g/L as $CaCO_3$)	2.02	0.17	b	2.04	0.12	b	2.87	0.14	a
VFA/ALK ratio	0.15	0.04	c	0.55	0.03	b	1.07	0.03	a
Acetate (g/L)	0.11	0.09	b	0.21	0.08	b	0.53	0.07	a
Propionate (g/L)	0.09	0.13	c	0.66	0.12	b	1.70	0.12	a
isoButyrate (g/L)	0.001	0.01	c	0.15	0.01	b	0.21	0.01	a
Butyrate (g/L)	0.003	0.01	c	0.13	0.01	b	0.17	0.01	a
isoValerate (g/L)	0.00	0.00	c	0.17	0.00	b	0.27	0.00	a
VS removal (%)	60.74	4.25	a	51.33	2.92	b	36.95	3.81	c
Digestate TS (%)	2.65	0.57	c	5.38	0.44	b	6.88	0.47	a
Digestate hemicellulose (%TS)	22.77	0.47	a	24.33	0.47	a	23.19	0.47	a
Digestate cellulose (%TS)	51.14	0.85	a	53.26	0.85	a	55.44	0.85	a

Note: SE: standard error. L: level (Tukey's HSD test, p-value < 0.05), different letters represent significant different groups, with highest value in group "a" and lowest in group "c". The darker the shading represents values further away from optimum range,

indicating signs of inhibition. Data from triplicated experiment with 5 measurements of each parameters at stable operating condition (n=15 for each condition). Valerate was not detected in all conditions.

4.3 Reactor instabilities and recoveries upon ORP-based micro-aeration

At OLR of 5 g VS/L/day, severe deteriorate in reactor performances indicated instability due to organic overloading. After the stable period reported in Table 4.1, the performance of reactors in all 3 replicated experiments began to deteriorate, indicated by key monitoring parameters identified in the previous section. Specifically, pH sharply plunged below 6.0, VFA/ALK ratio rapidly raised above 2 and up to 4 at some points, total VFA accumulated to over 9 g/L as HAc, and propionate exceeded 4 g/L in anaerobic reactors operated at OLR 5 g VS/L/day. Other parameters also showed signs of potential reactor failure, such as methane yield was recorded as low as 5 NmL/g VS added, CH_4 content was lower than 25% in biogas, and acetate concentration increased as high as 5 g/L.

All three reactors were on the verge of failure regardless of multiple alkalinity adjustment attempts, which provided only transient pH control (Figure 4.2, 4.3, and 4.4). Traditionally, under this circumstance, the substrate feeding need to be stopped and alkalinity need to be added to raise pH and buffering capacity of the reactor to facilitate the excess VFA consumption by methanogens. The cessation of the substrate feeding line and high cost of chemicals caused great logistic and economic impacts on the digesters. To solved this intrinsic problem of AD processes, we developed the ORP-based micro-aeration control system that allows the reactor to continue operate at the same OLR while quickly recovered to stable operating condition without the need for chemical addition. In the next sections, the performance of the ORP-based micro-aeration system in recovering reactor performance was evaluated in 3 replicated experiments.

4.3.1. The first replicated experiment

The bioreactor in the first experiment experienced instability from days 20 to 40 as the total VFA had increased to > 9 g HAc/L, the VFA/ALK ratio was > 2.0, and the pH had

dropped to < 5.5, resulting in a reduction in the methane yield as low as 10 NmL/g VS regardless of attempts to stabilize the bioreactor through alkalinity supplementations (Figure 4.2). Addition of $NaHCO_3$ could temporally raise the pH to 7 but has no effect on halting VFA accumulation and recover methane production. Thus, after stop alkalinity addition, the buffering capacity quickly depleted and unable to maintain neutral pH.

To overcome this limitation, the intermittent (24 h every other day) ORP-controlled micro-aeration operation was started on day 41. Within 12 days, the total VFA concentration decreased from 7.9 to 3 g HAc/L and the acetic acid concentration dropped from 4 g/L to less than 0.7 g/L with a concomitant increase in methane yield from 40 to 114 NmL/gVS. Compared to reactor under anaerobic condition, the total VFA reduced by 69% resulted in 303% enhance in methane yield under ORP-based micro-aerobic condition (Table 4.3). All other important parameters stabilized as well (Figure 4.2), suggesting a full recovery of the bioreactor.

Intermittent micro-aeration was achieved by controlling the ORP at a setpoint of -470 mV (+25 mV above the anaerobic baseline ORP of -495 mV) for a 24 h period, followed by a 24 h period without micro-aeration. The ORP levels varied from an average of -468 ± 4 mV to an average of -522 ± 12 mV, during days with and without micro-aeration, respectively, indicating a rapid exhaustion of residual DO and a switch back to typical anaerobic conditions once stopping micro-aeration. The ORP levels during anaerobic and micro-aerobic conditions were in close agreement with those in previous studies (Jenicek et al., 2011; Khanal and Huang, 2006). Thus, intermittent ORP-based micro-aeration allows for recovery from the verge of failure without supplementation of alkalinity and/or reducing the OLR.

4.3.2. The second replicated experiment

The duplicated experiment was performed to test the repeatability of the ORP-controlled micro-aeration system in controlling VFA and reactor stability. Similar trend of total VFA accumulation up to 10.9 g/L as HAc, resulted in methane yield as low as 5 NmL/g VS added at one point (Figure 4.3). On day 18, pH of the reactor felt below 5.5 and the VFA/ALK ratio raised over 4:1. This acidosis condition severely inhibited methanogens,

indicated by low CH_4 content in biogas (20% v/v) with accumulation of both acetate and H_2 (substrates of methanogens). From day 18 to day 40, multiple efforts to adjust pH and buffering capacity in the reactor were performed. Methane content in biogas was slightly recovered, resulting in slight increase in methane yield; however, total VFA concentration continue to steadily raise nonetheless.

On day 41, micro-aeration system was initiated by injecting oxygen into the reactor to maintain the average ORP of -450 mV (+25 mV from -475 mV, baseline ORP during anaerobic condition). Immediately, reductions of all 3 main VFA (acetate, propionate, and butyrate) were recorded, with acetate being the consumed at the fastest rate (0.25±0.1 g/day). After 15 days of intermittent micro-aerobic condition, total VFA reduced by half, from 10.9 to 5.2 g/L as HAc and stabilized after that.

From day 57 to 71, ORP set-point was increased to -425 mV (+50 mV from baseline ORP of -475 mV). This elevated ORP set point has negative effect on the performance of the reactor, causing slight accumulation of acetate during this period. This could be due to over aeration during this condition that inhibit strict anaerobic methanogens, resulted in the increase in acetate. Reduction in methane production at elevated ORP of higher than +50 mV compared to anaerobic ORP was also previously reported, possibly caused by excess substrate aerobic oxidation (Khanal and Huang, 2003). The effect was reversible after ORP set point for intermittent micro-aeration was reduced to -450 mV (+ 25 mV from baseline ORP) and the reactor stabilized until the end of the experiment. At the end of the experiment, acetate, propionate, and butyrate concentration was respectively reduced by 71.8%, 32.4%, and 91.9% compared to concentrations on day 40, prior to micro-aeration.

4.3.3. The third replicated experiment

The third replicated experiment validated the performance of the ORP-based micro-aeration process control system in reducing accumulated VFA and stabilizing AD of lignocellulosic biomass at organic overloading condition. During anaerobic condition, the total VFA concentration accumulated up to 9.6 g/L as HAc with acetate as the main component at 5.6 g/L (58% of total VFA). Upon intermittent micro-aeration by

controlling ORP level at -475 mV (+25 mV above anaerobic baseline ORP of -500 mV), the build-up VFA were quickly reduced to maintain stable pH and VFA/ALK ratio without addition of alkalinity. After 14 days of intermittent ORP-based micro-aeration, the total VFA concentration reduced to stable level of 2.5 g/L as HAc, with 92%, 44.9%, and 88.5% reduction in acetic, propionic, and butyric acid, respectively. On day 66, the micro-aeration was stopped and the ORP returned to anaerobic baseline level of -508 ± 3 mV. Under anaerobic condition, the bioreactor experienced accumulations of VFA again, indicating the intrinsic instability of AD at high OLR and merits of the micro-aeration process in controlling low VFA accumulation and overall stability of the process.

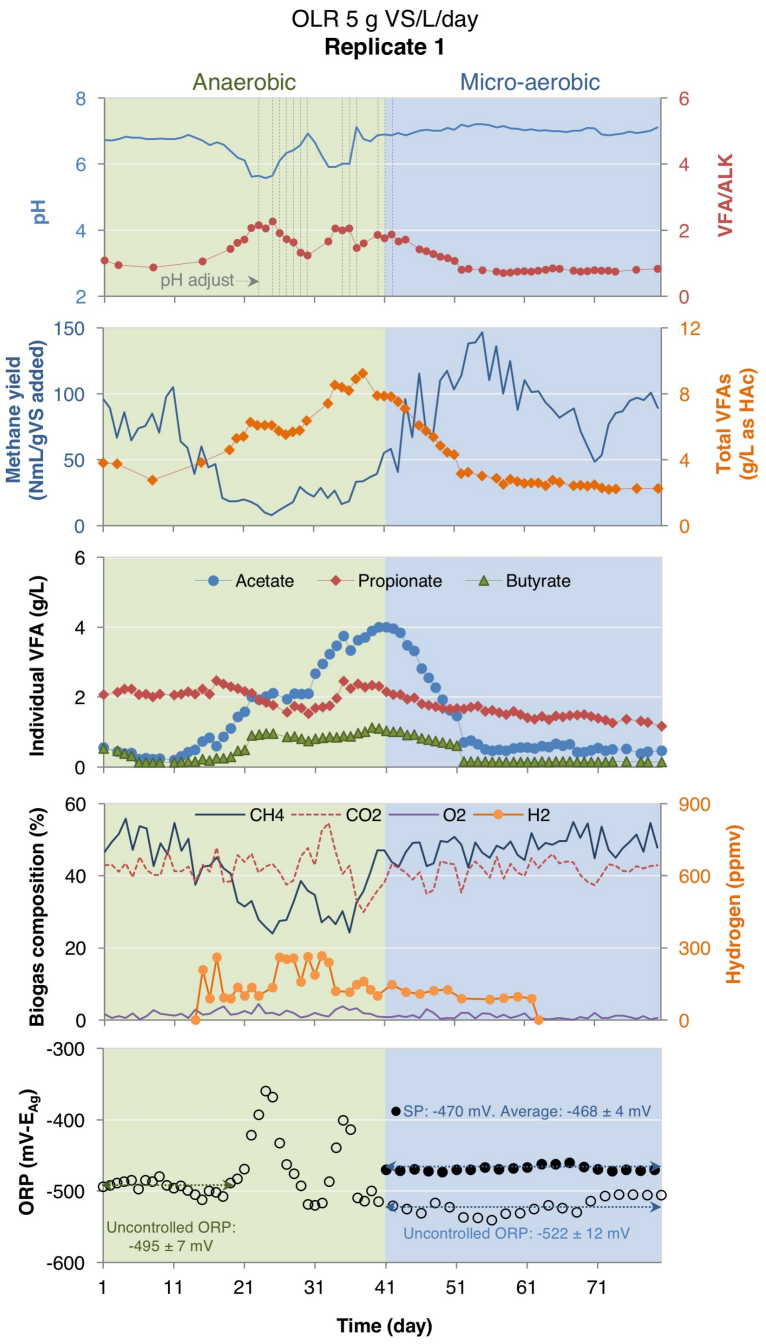

Figure 4.2 Reactors performance in anaerobic and ORP-controlled micro-aerobic condition at OLR 5 g VS/L/day in first replicated experiment.

Note: The monitoring period is trimmed to 40 days before and after the micro-aeration initiation. The long-term performance of this reactor is presented in Figure B.2.

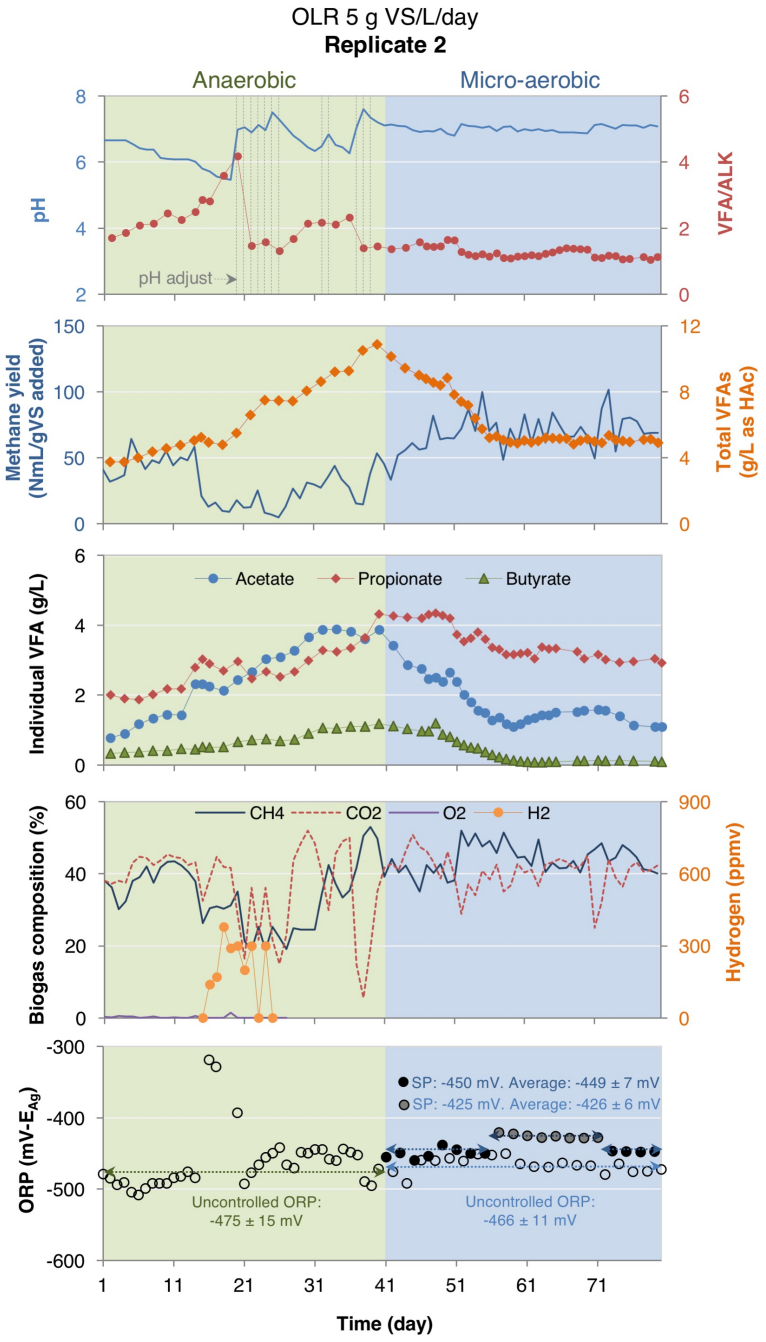

Figure 4.3 Reactors performance in anaerobic and ORP-controlled micro-aerobic condition at OLR 5 g VS/L/day in second replicated experiment.

Note: The monitoring period is trimmed to 40 days before and after the micro-aeration initiation. The long-term performance of this reactor is presented in Figure B.3

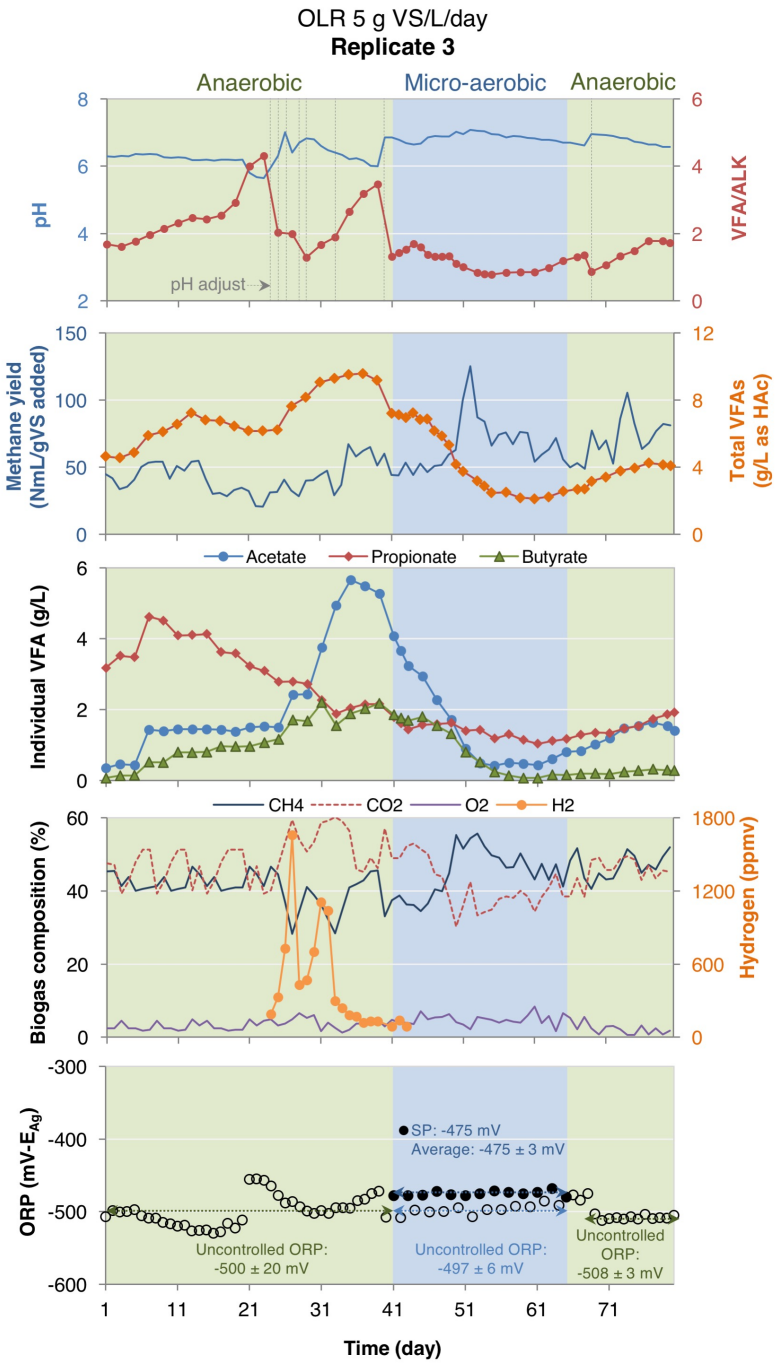

Figure 4.4 Reactors performance in anaerobic and ORP-controlled micro-aerobic condition at OLR 5 g VS/L/day in third replicated experiment.

Note: The monitoring period is trimmed to 40 days before and after the micro-aeration initiation. The long-term performance of this reactor is presented in Figure B.4

4.3.4. Overall performance of the ORP-based micro-aeration system

Considering data from triplicated experiment, statistical analysis confirmed that the ORP-based micro-aeration process control system significantly reduced all types of VFA concentration, maintained pH and VFA/ALK ratio at desirable range, and recovered methane yield and VS reduction of the bioreactors (Figure 4.5, Table 4.2).

The study shown that alkalinity supplementation could not control the stable performance of AD reactors at high OLR and eventually led to severe process inhibition caused by over accumulation of VFA. The ORP-based micro-aeration in this study demonstrated rapid and reliable ability to recover the reactor from the verge of failure without the need of supplementing alkalinity or reducing OLR.

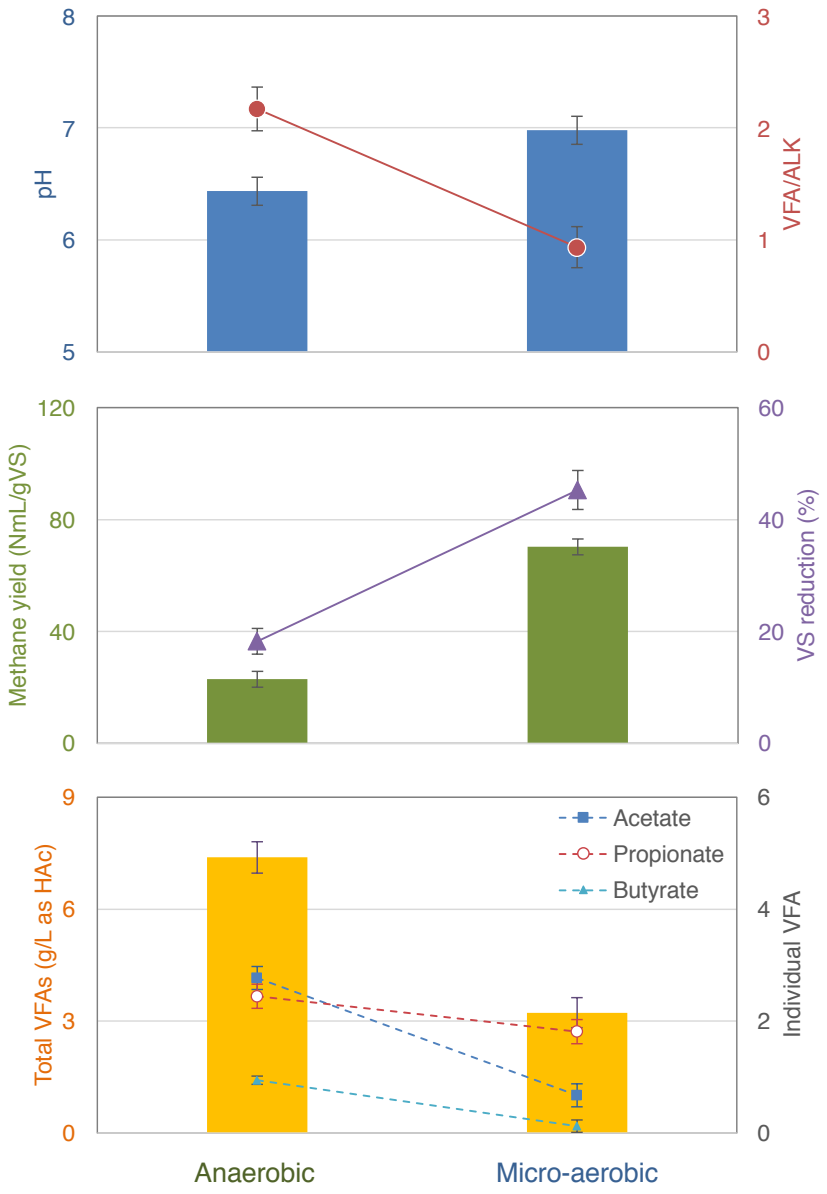

Figure 4.5 Comparison of reactor performances at anaerobic and ORP-based micro-aerobic condition.

Note: Data shown as mean and standard error from triplicated experiments with 5 measurements of each parameters (n=15 for each condition). All data were significant different between anaerobic and micro-aerobic condition (ANOVA test, $p<0.001$).

Table 4.2 Reactors performance at OLR 5 g VS/L/day during different stages

	Stages of reactors at OLR 5 g VS/L/day								
	Anaerobic stable			Anaerobic unstable			Micro-aerobic		
	Mean	SE	L	Mean	SE	L	Mean	SE	L
pH	6.73	0.12	b	6.43	0.12	c	6.98	0.12	a
ORP (mV-E_{Ag})	-503	5	b	-465	5	a	-463	5	a
ORP (mV-$E_{h\ pH\ 7}$)	-318	12	c	-297	12	b	-263	12	a
Methane yield (NmL/gVS added)	80.95	2.83	a	22.9	2.83	b	80.84	2.83	a
CH_4/CO_2 ratio	1.03	0.04	a	0.72	0.04	b	1.10	0.04	a
CH_4 (% v/v)	47.41	2.59	a	31.22	2.59	b	47.06	2.59	a
CO_2 (% v/v)	46.76	4.20	a	46.01	4.20	a	42.95	4.20	a
O_2 (% v/v)	1.17	1.09	b	2.10	1.09	a	1.99	1.09	ab
Total VFA (g /L as HAc)	3.04	0.46	b	7.39	0.42	a	3.23	0.40	b
Total alkalinity (g/L as $CaCO_3$)	2.87	0.41	b	3.89	0.37	a	3.49	0.35	ab
VFA/ALK ratio	1.07	0.22	b	2.17	0.19	a	0.93	0.18	b
Acetate (g/L)	0.53	0.20	b	2.77	0.20	a	0.67	0.2	b
Propionate (g/L)	1.70	0.21	b	2.44	0.21	a	1.81	0.21	b
isoButyrate (g/L)	0.21	0.02	a	0.17	0.02	b	0.09	0.02	c
Butyrate (g/L)	0.17	0.07	b	0.94	0.07	a	0.12	0.07	b
isoValerate (g/L)	0.27	0.03	a	0.25	0.03	a	0.03	0.03	b
Valerate (g/L)	0.09	0.03	b	0.13	0.03	a	0.05	0.03	c
VS removal (%)	36.95	2.00	a	18.24	2.30	b	45.28	3.48	a
Digestate TS (%)	6.88	0.55	b	10.89	0.55	a	8.88	0.58	a

Note: Anaerobic stable stage: first 5 days of anaerobic condition; anaerobic unstable stage: last 5 days of anaerobic condition (prior to micro-aeration started); micro-aerobic stage: last 5 days of micro-aerobic condition. SE: standard error. L: level (Tukey's HSD test, p-value < 0.05), different letters in each row represent significant different groups, with highest value in group "a" and lowest value in group "c". The darker the shading represents values further away from optimum range, indicating signs of inhibition. Data from triplicated experiment with 5 replicate measurements of each parameters at stable operating condition (n=15 for each condition).

Table 4.3 Comparison on effects of micro-aeration on VFA concentration and methane yield in different studies

Main purpose	Anaerobic ORP (mV)	Micro-aerobic ORP (mV)	Effect on total VFA	Effect on methane yield	Reference
H_2S removal	-300 to -290	-275 to -265	NA	-15.5% (1g/L SO_4^{2-}) -6.2% (3 g/L SO_4^{2-}) + 45.9% (6g/L SO_4^{2-})	(Khanal et al., 2003)
H_2S removal	-280	-230	NA	- 66.7% (1g/L SO_4^{2-}) +56.3% (5 g/L SO_4^{2-})	(Khanal and Huang, 2003)
H_2S removal	-280	-180	NA	-96.3% (1g/L SO_4^{2-}) +57.9% (5 g/L SO_4^{2-})	(Khanal and Huang, 2003)
Enhance sludge quality	-347	-307	NA	-3.7%	(Jenicek et al., 2014)
Enhance hydrolysis	-97	-100	+43%	NA	(Lim et al., 2014)
Enhance hydrolysis	-97	-172	+32%	NA	(Lim et al., 2014)
Enhance hydrolysis and methane	-253 to -270	-220 to -278	+300%	+21%	(Lim and Wang,

yield					2013)
Reduce VFA concentration	-295	-268	-69.7%	+303%	This study (Rep. 1)
	-275	-249	-33.8%	+300%	This study (Rep. 2)
	-300	-275	-64.5%	+157%	This study (Rep. 3)

Note: ORP values with SHE reference electrode (E_h). Reduction (-) and increase (+) in total VFA concentration and methane yield comparing micro-aerobic to anaerobic condition. Data in this study from triplicated experiment with 5 replicate measurements of each parameters at stable operating condition (n=15 for each condition). NA: not available.

4.4 Controlling ORP through micro-aeration in the AD process

In this study, micro-aerobic conditions were maintained via ORP-controlled micro-aeration at a target ORP of +25 mV above the baseline ORP of an anaerobic bioreactor. For instance, the micro-aerobic ORP was controlled at -470 mV, -450 mV, and -475 mV in the first, second, and third set of experiment, respectively. The daily average ORP was precisely controlled at desired value (< 2 mV off set from set point) by injecting oxygen when ORP level felt 10 mV below the target value (Figure 4.6). The injected oxygen promptly spiked up the ORP to approximately 10 mV above the target value, which then prompted the solenoid valve to close thereby stopping the micro-aeration. Hence, a uniform ORP profile oscillating between 10 mV below and above the target ORP was obtained (Figure 4.6).

To reduce accumulated VFA, controlling ORP (E_{Ag}) at -475 and -470 mV (E_h =-275 to -270 mV) showed greater performance than the more oxidative ORP micro-environment at -450 mV (Table 4.3). Also, elevated ORP at -425 mV (i.e. +50 mV above the baseline anaerobic level) in the second experiment caused slight increase in acetate concentration in the reactor (Figure 4.3). This was putatively resulted from the overdosing of oxygen at micro-aeration rate of 477 ± 222 mL O_2/day at elevated ORP of -425 mV, as compared to

only 102 ± 59 mL O_2/day at ORP setpoint of -450 mV (Figure 4.7). This surplus amount of supplied oxygen might not be completely consumed by facultative bacteria and hence inhibited the activity of anaerobic aceticlastic methanogens; however, the overall methane yield did not show sign of reduction during this period.

In addition, the ORP-controlled micro-aerobic condition was maintained during 24 h followed by 24 h of uncontrolled ORP. During this uncontrolled ORP day, the ORP quickly reduced to -508 to -522 mV, indicating the anaerobic condition and rapid depletion of oxygen once micro-aeration stopped. However, in the second replicated experiment, the uncontrolled ORP remained relatively high at -466 ± 11 mV (Figure 4.3). Previous studies (Table 4.3) also mentioned that micro-aeration at elevated ORP of +50 and +100 mV from anaerobic baseline ORP could resulted in methane yield reduction due to excess aerobic respiration by facultative heterotrophs. This was probably due to higher oxidative micro-environment that was unfavorable for co-existence of facultative bacteria and anaerobic methanogens (Liu et al., 2013). Thus, 24 h cycle intermittent micro-aeration controlled at ORP of +25 mV above anaerobic ORP level was sufficient to control the VFA concentration and stabilizing process performance.

Intermittent aeration with fluctuating ORP was previously applied for nitrogen removal by creating sequential anoxic-aerobic cycles (Lackner et al., 2012; Ma et al., 2017; Shi et al., 2013). Controlling specific ORP level was also studied to increase yield of specific anaerobic and aerobic fermentation products (Liu et al., 2013). This is the first study that shows ORP-controlled micro-aeration could reduce VFA accumulation and enhance methane yield, likely by creating condition for both niches of facultative bacteria and anaerobic methanogens.

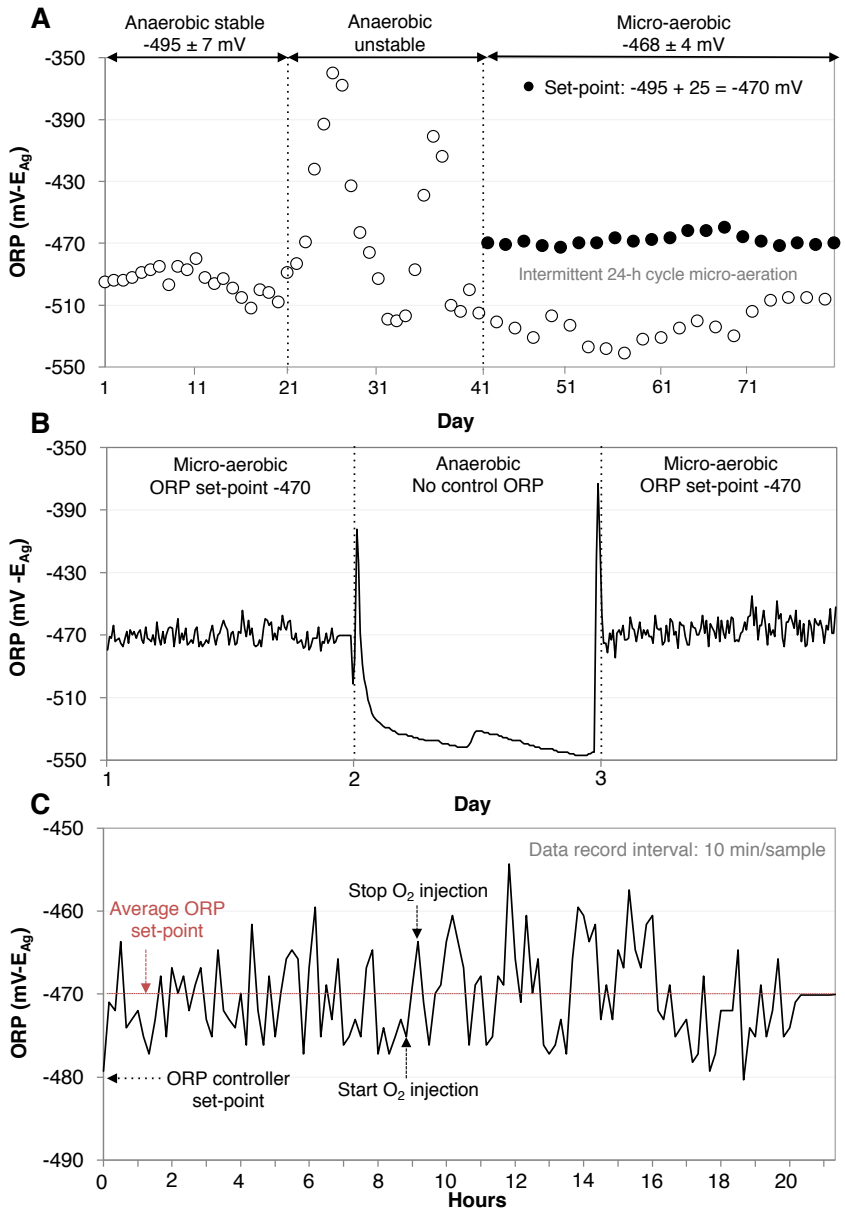

Figure 4.6 The ORP profile of bioreactor 1 under anaerobic uncontrolled ORP and intermittent ORP-controlled micro-aerobic condition.

Note: Daily average ORP of uncontrolled ORP (open circles) and controlled ORP (closed circles) (A); ORP profile at micro-aerobic condition for three days (day 41-43 in section A): cycle of 24 h micro-aeration followed by 24 h pause (B); ORP profile at micro-aerobic condition for one day (day 1 in section B) with data recorded every 10 min, ORP

controller setpoint: value at which solenoid valve open to start injecting oxygen, average ORP setpoint: target ORP value (C).

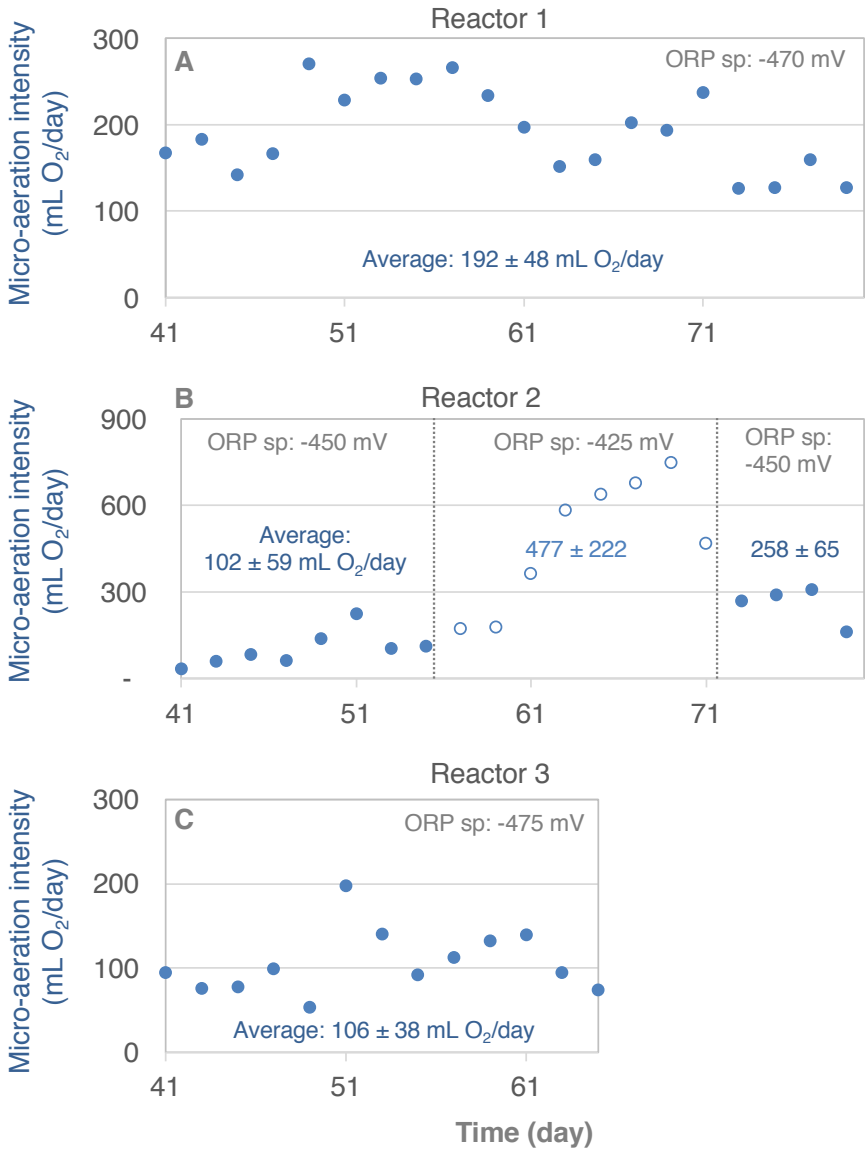

Figure 4.7 Micro-aeration intensity of 3 reactors during ORP-based micro-aerobic conditions.

4.5 Mass balance of reactors during anaerobic and micro-aerobic condition

The results of carbon mass balance in reactors before and after micro-aeration indicate that micro-aerobic condition enhanced carbon recovery through lignocellulosic degradation and VFA conversion. Specifically, the digestate from reactors during micro-aerobic condition consisted of 2.84 ± 0.13 g carbon, which was significantly lower (p<0.05) than 3.66 ± 0.13 g carbon in the digestate without micro-aeration (Figure 4.8). Examination from SEM pictures showed clear contrast between the smooth surface of raw Napier grass with rough surface of partially digested fibers from AD reactors under anaerobic and micro-aerobic condition (Figure 4.9). Visually, the fiber structure of micro-aerobic digested grass was disrupted more than fibers taken from reactor under anaerobic condition, suggesting a higher hemicellulose and cellulose degradation upon micro-aeration. Fiber analyses were therefore performed to confirm this hypothesis. Results showed that the digestate from reactors during micro-aerated condition consisted significantly lower hemicellulose content than in the digestate before micro-aeration (Table 4.4). This information was further supported by the increase in VS reduction from 18.24% during anaerobic unstable period to 45.28% after micro-aeration was initiated (Table 4.2).

The ability to promote degradation of hemicellulose in lignocellulosic biomass suggests that micro-aeration can be used as a strategy to facilitate hydrolysis of difficult to degrade AD substrates. In recent years, micro-aeration was successfully applied as biological pre-treatment method to enhance hydrolysis of recalcitrant substrates that speed up this rate limiting step of AD processes (Fu et al., 2014, 2016; Lim and Wang, 2013; Sawatdeenarunat et al., 2017). The unanimous results from these studies proved that micro-aeration promoted the excretion of extracellular hydrolytic enzymes to increase the hydrolysis of various substrates, resulted in more soluble substrate availability and methane production. Thus, results from this research provide important additional information that proved enhancing hydrolysis using micro-aeration could be possible in semi-continuous mix-cultured CSTRs.

This increase in substrate degradation resulted in higher soluble substrate for microbial community and therefore significantly improved biogas production volume and therefore carbon recovery in the forms of CO_2 and CH_4 (Figure 4.8). In addition, the removal of accumulated VFA stabilized pH of the reactors and instantly recovered activities of methanogens, resulted in significant enhancement in CH_4 (p<0.005). As a result, the ratio of CH_4/CO_2 in biogas was recovered from 0.7 to 1.1 ± 0.04 after micro-aeration. However, under the effect of micro-aeration, only the CH_4 content in biogas was increased (p<0.05), but not the CO_2 composition (p>0.05) (Table 4.2). This finding suggests that the ORP-based intermittent micro-aeration created an environment for hydrogenotrophic methanogens to utilized the excess CO_2 produced from the aerobic oxidation of VFA by facultative heterotrophs. Hence, we hypothesized that the niches of facultative heterotrophs and anaerobic methanogens could work together in such ORP controlled environment to convert excess VFA into CO_2 and then to CH_4, resulted in a stable performance even at high OLR.

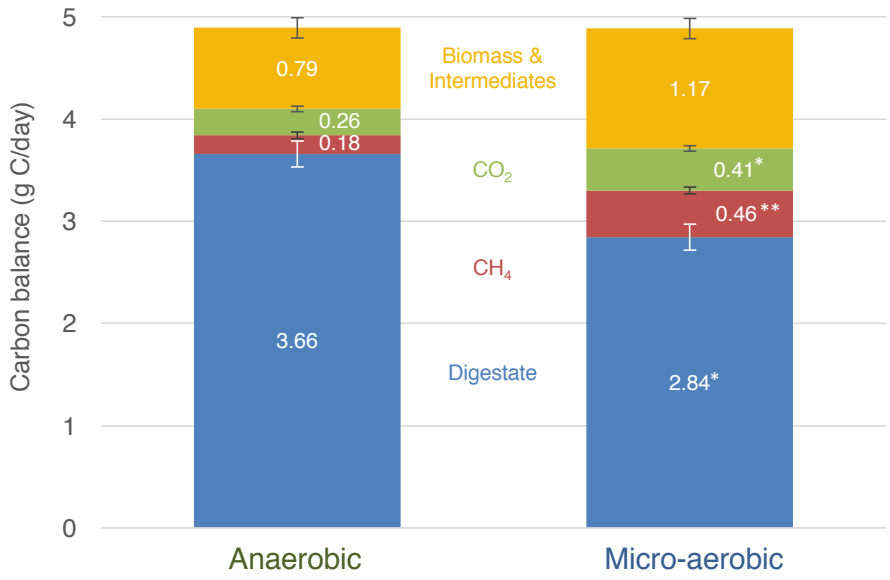

Figure 4.8 Carbon mass balance during anaerobic and micro-aerobic conditions.

Note: Data present as mean value and standard error of triplicated samples from 3 replicated experiments (n=9 for each condition). Significant difference from ANOVA test with p<0.05 (*) and p<0.005 (**).

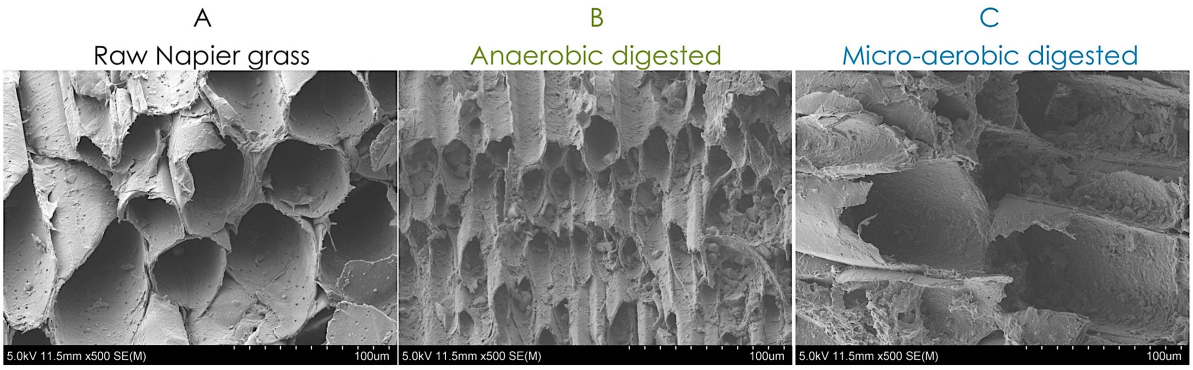

Figure 4.9 Scanning electron microscope (SEM) pictures of raw Napier grass feedstock (A), digested fibers from the first reactor under anaerobic (B) and micro-aerobic condition (C).

Table 4.4 Carbon mass balance and digested fiber composition at anaerobic and micro-aerobic condition.

	Components	**Anaerobic**	**Micro-aerobic**	***p*-value**
Carbon mass balance (g C/day)	Digestate	3.66 ± 0.13	2.84 ± 0.13	0.011*
	CH_4	0.18 ± 0.03	0.46 ± 0.03	0.004**
	CO_2	0.26 ± 0.03	0.41 ± 0.03	0.017*
	Biomass & Intermediates	0.79 ± 0.10	1.17 ± 0.10	0.054
Digested fiber composition (%TS)	Hemicellulose	22.96 ± 0.59	22.41 ± 0.59	0.003**
	Cellulose	55.15 ± 0.47	54.22 ± 0.47	0.058
	Acid detergent lignin	7.64 ± 0.35	8.35 ± 0.35	0.239
	Extractive	9.05 ± 0.71	9.40 ± 0.71	0.500
	Ash	5.20 ± 0.20	5.61 ± 0.20	0.167

Note: For each condition, carbon mass balance data present as mean ± standard error (n=9), digested fiber composition data present as mean ± standard error (n=6). Significant difference from ANOVA test with $p<0.05$ (*) and $p<0.005$ (**).

4.6 Bioenergetics of anaerobic and micro-aerobic VFA conversion pathways

To better understand the mechanisms of VFA conversion, thermodynamic calculations were performed for micro-aerobic and anaerobic conditions in 3 replicated reactors (Figure 4.10, Table 4.5). As expected, results show that the Gibbs free energies associated with complete VFA oxidation to CO_2 using O_2 as the electron acceptor ($\Delta G'$ = -92.1 to -88.2 kJ/e^-) were much higher than those for incomplete VFA fermentation coupled with proton reduction ($\Delta G'_R$ = -14.8 to 5.0 kJ/e^-). The calculations show that under both standard condition and actual reactors conditions, the VFA aerobic oxidation reaction are much more thermodynamically desired over anaerobic fermentation of VFA that need to work in syntrophy with hydrogenotrophic methanogens (Table 4.5). Taken together, these results suggest that facultative anaerobes switched from anaerobic fermentation to the more thermodynamically and kinetically favorable aerobic respiration (Table 2.3, Table 4.5). The availability of O_2, even at very low concentrations, can redirect the metabolism of facultative bacteria towards the more energetically favorable aerobic respiration pathway by regulating expression of aerobic metabolism related genes (Forster and Gescher, 2014; Morris and Schmidt, 2013). Thus, VFA produced during the 24 h without micro-aeration were likely consumed by facultative heterotrophs when micro-aeration was provided, resulting in low VFA concentrations during the period with intermittent micro-aeration. While alkalinity addition during anaerobic operation only caused transient relieve of inhibition for methanogens, intermittent micro-aeration effectively reduced VFA by promoting their aerobic oxidation by facultative heterotrophs.

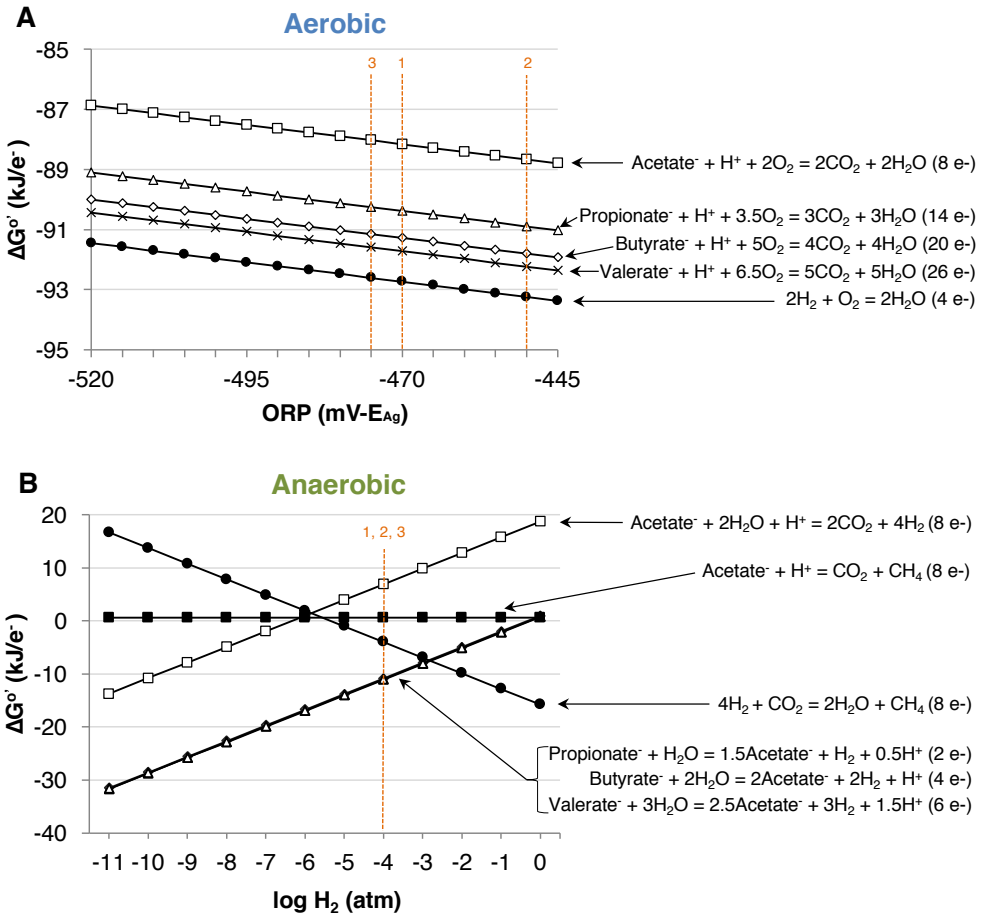

Figure 4.10 Thermodynamic comparison of VFA consumption pathway under micro-aerobic and anaerobic condition.

Note: Gibbs free energy change ($\Delta G^{o'}$) of aerobic reactions as function of ORP (A) and anaerobic reactions as function of H_2 partial pressures (B). Legends are stoichiometric equations with the number of electron transferred in parentheses. Calculations were performed according to (Thauer et al., 1977) with all reactions in the aqueous phase and based on conditions on day 40 (immediately before micro-aeration began), indicated by orange vertical lines with the number represents the 1st, 2nd and 3rd replicated experiment.

Table 4.5 Thermodynamic of reactions in reactors at anaerobic and micro-aerobic condition

No.	Reactions	$\Delta G^{o'}$ (kJ/e^-)	$\Delta G'$ (kJ/e^-)		
			R1	R2	R3
Anaerobic condition					
	$Valerate + 3\ H_2O = 2.5\ Acetate + 3\ H_2 + 1.5\ H^+$	20.88	-10.85	-13.61	-10.02
	$Butyrate + 2\ H_2O = 2\ Acetate + 2\ H_2 + H^+$	20.87	-11.05	-13.99	-10.73
	$Propionate + 1\ H_2O = 1.5\ Acetate + H_2 + 0.5\ H^+$	20.90	-10.99	-14.75	-10.03
	$Acetate + H^+ + 2\ H_2O = 2\ CO_2 + 4\ H_2$	22.69	4.57	2.24	4.99
Micro-aerobic condition					
	$Valerate + H^+ + 6.5\ O_2 = 5\ CO_2 + 5\ H_2O$	-109.19	-91.72	-92.14	-91.42
	$Butyrate + H^+ + 5\ O_2 = 4\ CO_2 + 4\ H_2O$	-109.14	-91.29	-91.73	-91.10
	$Propionate + H^+ + 3.5\ O_2 = 3\ CO_2 + 3\ H_2O$	-109.03	-90.38	-90.91	-90.10
	$Acetate + H^+ + 2\ O_2 = 2\ CO_2 + 2\ H_2O$	-108.78	-88.16	-88.48	-87.96
	$2\ H_2 + O_2 = 2\ H_2O$	-131.46	-92.73	-90.73	-92.94
Methanogenesis					
	$Acetate + H^+ = CO_2 + CH_4$	-1.42	0.60	0.81	0.58
	$CO_2 + 4\ H_2 = 2\ H_2O + CH_4$	-24.10	-3.97	-1.43	-4.40

Note: Change in Gibbs free energy at standard condition (25^{o}C, pH 7, 1 M and 1 atm) ($\Delta G^{o'}$, kJ/electron equivalent) and at reactors condition on day 40 prior to the start of micro-aeration ($\Delta G'$, kJ/electron equivalent) were calculated as described in (Dolfing, 2015; Thauer et al., 1977) with all components in the aqueous phase.

4.7 Changes in microbial community structure under the effect of ORP-controlled micro-aeration

Bacterial and archaea community structure was constructed by sequencing bacterial 16S rRNA gene and *mcrA* gene, respectively and the results are shown in Figure 4.11 and Table B.1. Bacterial community structure of reactor under anaerobic condition consisted of 4 main phyla *Bacteriodetes*, *Firmicutes*, *Proteobacteria*, and *Spirochaetae* with 39%, 21% 7%, and 2% of total sequence reads, respectively. There are total of 25 bacterial operational taxanomic unit (OTUs) with relative abundance at genus level > 1% of the total sequence reads. Among them, 9 OTUs belong to *Bacteriodetes*, 11 OTUs are *Firmicutes,* 4 OTUs are *Proteobacteria,* and 1 belong to *Spirochaetae.* Bacteria affiliated to *Bacteriodetes* and *Firmicutes*, with relative abundance of 39% and 21%, respectively, were also found as dominant groups in AD reactors fed with lignocellulosic biomass (Fu et al., 2014; Sundberg et al., 2013). Metaproteomic studies also confirmed that *Fimicutes* and *Bacteroidetes* phylum are responsible for the hydrolysis and fermentation of hemicellulose, cellulose and other polysaccharides, while *Proteobacteria* are mainly glucose and VFA utilizing bacteria (Ariesyady et al., 2007; Hanreich et al., 2013).

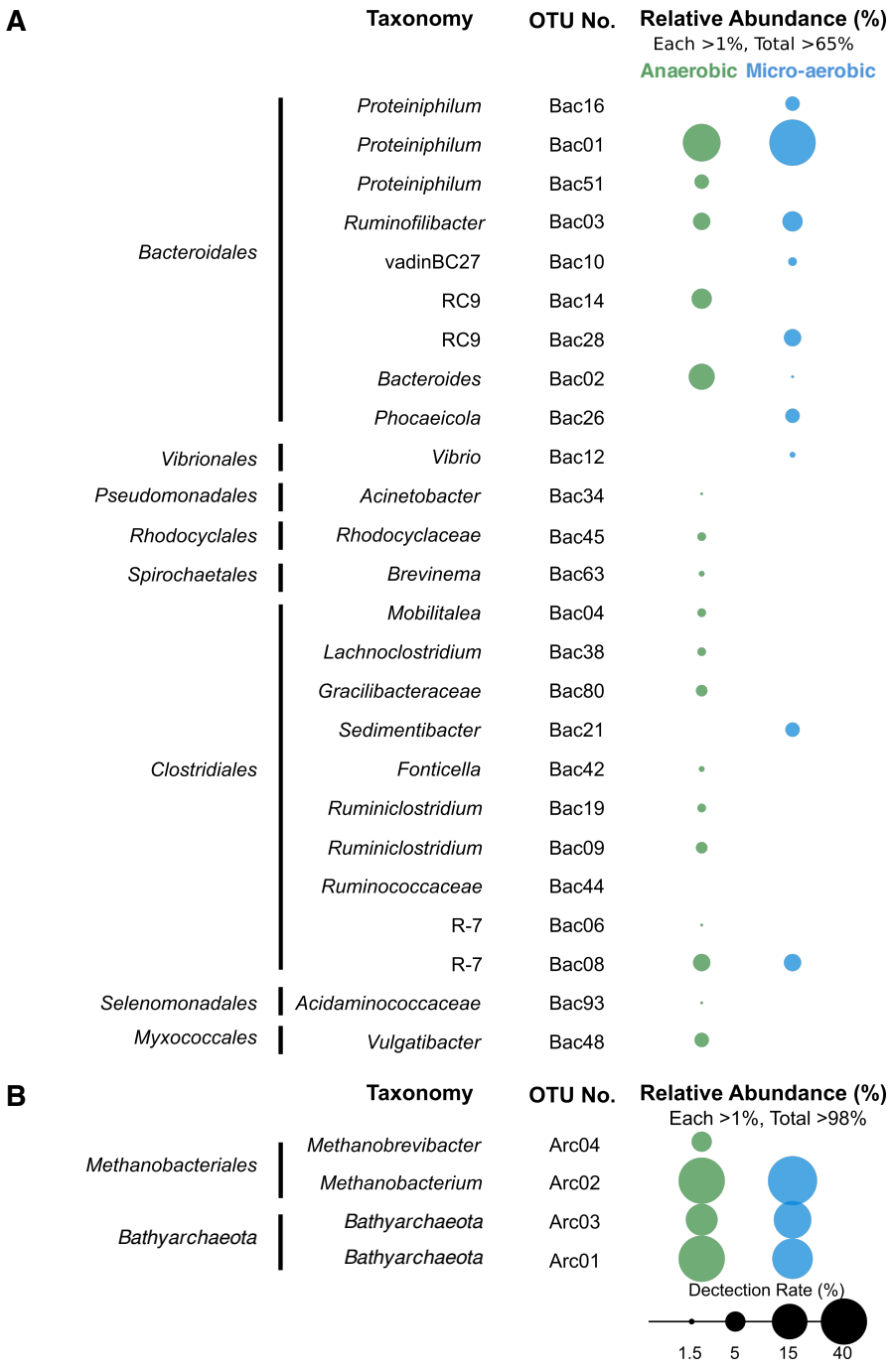

Figure 4.11 Bacterial community structure (A) and archaeal community structure (B) without (day 21) and with intermittent ORP-controlled micro-aeration (day 71) in reactor 1 (Figure 4.2).

Note: Details of relative abundance and potential function of identified OTUs are presented in Table B.1

The dominant OTUs before and after initiation of micro-aeration were found to be facultative anaerobes (Table B.1). The relative abundance of OTUs representing the genus *Proteiniphilum* increased from 21.2% to 44.3% after implementing micro-aeration (Figure 4.11 and Table B.1). In addition, there are no syntrophs among dominant OTUs (relative abundance >1% at genus level) in reactors under both conditions, suggesting the lack of syntrophic acetogenesis. This microbial data further confirmed the hypothesis suggested by reactor performance and thermodynamic calculations that facultative bacteria were likely to aerobic oxidized VFA under ORP-controlled micro-aerobic condition, bypassing the thermodynamic constrained syntrophic acetogenesis pathway.

Intermittent ORP-based micro-aeration created aerobic niches for VFA conversion by facultative microbes, it also promoted methanogenesis as shown by the increased production of methane (Figure 4.2, Figure 4.3, and Figure 4.4). Even though the accumulated VFA were likely oxidized to CO_2 through aerobic respiration during micro-aeration, there was no significant change (p>0.05) in the CO_2 content of the biogas (Table 4.2), suggesting that CO_2 was reduced to CH_4 using H_2 as the electron donor. Archaeal community analysis from *mcrA* gene sequencing confirmed this hypothesis by showing that all methanogens identified (*Methanosarcina*, *Methanobacterium*, and *Methanospirillum*) in reactors under both anaerobic and micro-aerobic condition can perform hydrogenotrophic methanogenesis (Table B.1). Some species of these methanogens can produce antioxidative enzymes, allowing them to survive under oxidative stress condition (Brioukhanov et al., 2006; Horne and Lessner, 2013). Dominant of hydrogenotrophic methanogens in was previous mentioned in various micro-oxic environments, such as in flooding rice field soils (Liu and Whitman, 2008) or micro-aerated AD reactor (Fu et al., 2016).

While H_2 oxidation with O_2 to H_2O ($\Delta G'$ = -92.7 kJ/e^-) is thermodynamically more favorable than H_2 reduction to CH_4 ($\Delta G'$ = -4.0 kJ/e^-), the supplied O_2 was likely completely consumed during VFA oxidation, leaving the available H_2 for hydrogenotrophic methanogens. Although the Gibbs free energies for aerobic oxidation of VFA and H_2 are comparable ($\Delta G'$ = -88.2 to -92.7 kJ/e^-), the fast-growing facultative heterotrophs (μ_m = 13.2 day^{-1}) had an advantage in the presence of excess VFA over the

slow-growing H_2-oxidizing bacteria (μ_m = 10.1 day^{-1}) with limited H_2 availability (Table 2.3). Consistent with this, OTUs associated with facultative heterotrophs accounted for 44.3% of the total bacteria while H_2-oxidizing bacteria were not detected among the dominant OTUs (Table B.1).

With the intermittent ORP-controlled micro-aeration, increase in H_2 and CO_2 productions from enhanced fermentation and VFA aerobic oxidation, respectively (Figure 4.8), created a reaction quotient to thermodynamically favor hydrogenotrophic methanogenesis (Dykstra and Pavlostathis, 2017). Besides, rapid conversion of accumulated VFA during micro-aeration could also rejuvenated aceticlastic methanogenesis as supported by the high relative abundance of OTUs representing genus *Methanosarcina* (Figure 4.11 and Table B.1). Dominant of *Methanosarcina* in reactors could be explained by their robust under high VFA and oxidants concentrations condition and ability to produce methane from both acetate and H_2 + CO_2 (De Vrieze et al., 2012; Horne and Lessner, 2013). Increase in relative abundance of oxytolerant methanogens *Methanosarcina* in micro-aerobic reactor was also mentioned in other research as archaea adaptation mechanism to micro-oxic environment (Fu et al., 2016). Therefore, intermittent ORP-controlled micro-aeration provided O_2 as the limiting electron acceptor for facultative heterotrophs allowing partial consumption of VFA while maintaining an environment suitable for aceticlastic and hydrogenotrophic methanogens.

4.8 Proposed metabolic pathway of lignocellulosic biomass digestion via intermittent ORP-based micro-aerobic condition

ORP-controlled intermittent micro-aeration promoted degradation of lignocellulosic biomass and VFA conversion bypassing syntrophic acetogenesis by facultative bacteria while conserving crucial anaerobic niches for aceticlastic and hydrogenotrophic methanogenesis. The resulting balance between anaerobic and facultative microbes allowed for stable operation at high OLR. Combining reactor performance results, mass balance analyses, microbial community characterization data, and a bioenergetics evaluation, we propose a methane producing pathway from lignocellulosic biomass at high OLR under intermittent ORP-controlled micro-aerobic condition as illustrated in Figure 4.12. Metatranscriptomic analyses to evaluate specific transcriptional activities

during anaerobic and aerobic respiration of this complex microbial community are needed to confirm the proposed pathway. This newly identified operational strategy can be applied to operate anaerobic digesters at high OLRs without the need for chemical addition for pH control, and has significant economic and logistical merits for full-scale implementation of anaerobic digestion of lignocellulosic biomass.

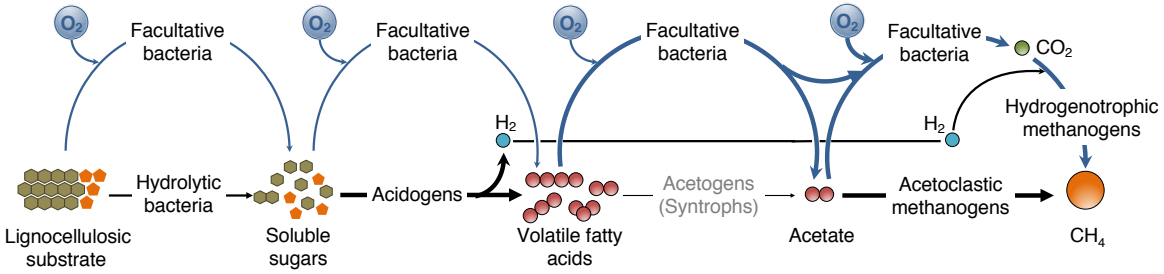

Figure 4.12 Proposed methane producing pathway under intermittent ORP-based micro-aerobic condition

Chapter 5

Engineering implications

An innovative ORP-based micro-aeration process control system to rapidly recover AD reactors from the verge of failure and maintain stable performance at high organic loading rates was developed and validated in this study. This unique control system proved to be effective in reducing VFA concentration, stabilizing pH, and enhancing methane production, allowing AD reactors to be continuously operated at OLRs, which would normally cause organic overloading disturbance. As a result, feedstocks accumulations could be prevented and the continuous process flow could be maintained. In addition, adopting this technology could reduce or eliminate the requirement of chemical addition for controlling pH in anaerobic digesters. Hence, this process control technology offers significant economic and logistical merits for AD plants.

The simple design and operating principle of this ORP-based micro-aeration system makes it easy to implement in existing anaerobic digesters as a "plug-and-play" process control technology. The robustness of ORP probe allows long term operation of this ORP-based micro-aeration system even in high solid AD processes such as AD of lignocellulosic biomass. Since majority of co-digestion or mono-digestion of lignocellulosic biomass are CSTRs (Khanal, 2008), the system developed in this study could be readily applied in those anaerobic digesters. Only minor modifications are required to apply this technology, such as installation of a low flowrate air pump, ORP probes and controller (already available in many AD plants), and slightly increase mixing speed for better air distribution. Multiple methods of micro-aeration could be implemented (e.g. injecting air/oxygen in the bottom of the reactor, feeding line, substrate mixing tank, or by adding aerated water), allowing the flexibility of applying this micro-aeration process control in existing AD plants. The small capital and operational cost makes this technology affordable even for small-scaled anaerobic digesters. Figure 5.1 illustrates an example of the design of this ORP-based micro-aeration process control system in an industrial scale anaerobic digester. Anaerobic digesters with capacity of less than 1,000 m^3 are recommended for the application of this micro-aeration process control

technology at this stage. Application of micro-aeration on larger digesters might face some challenges from oxygen transfer and homogeneous distribution. To overcome this problem, a specific micro-aeration system with air diffusion ring and mixing paddle could be installed in new anaerobic digesters (Figure 5.1). Different engineering considerations for designing and operating the ORP-based micro-aeration control system need to be examined and adjusted accordingly, as discussed in detail in section 2.3.

With the rapid growing trend of micro-aeration researches and applications in AD processes in recent years, such as to remove H_2S or enhance hydrolysis and now to stabilize the process at high OLR, the widely implementation of micro-aeration system in AD plants could be expected in the next 10 years.

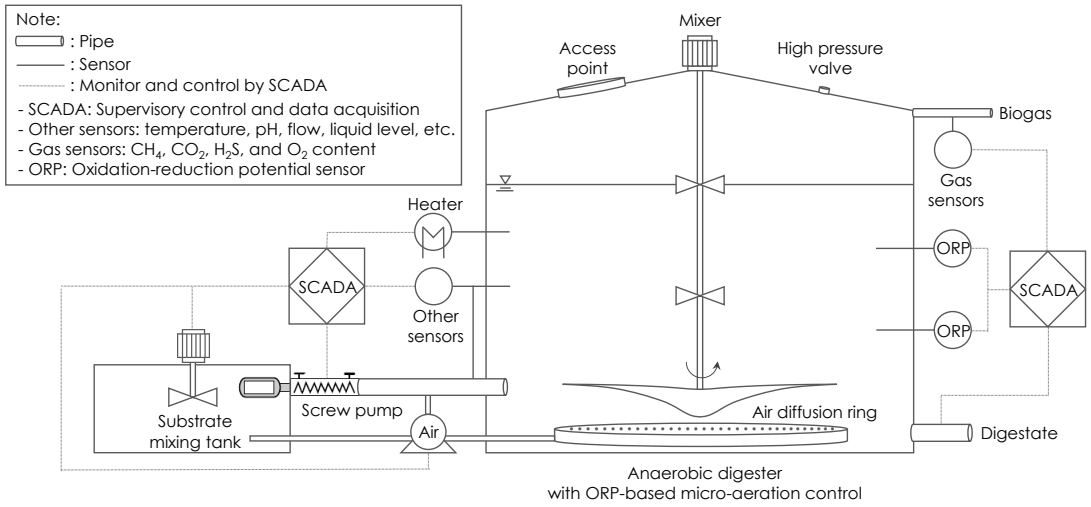

Figure 5.1. Example of an industrial scale anaerobic digester with ORP-based micro-aeration process control

Chapter 6

Conclusions and Recommendations

6.1 Conclusions

Controlling VFA accumulation and stable methane production have been major challenges for anaerobic digesters operated at high OLRs due to kinetic and thermodynamic imbalance between VFA-producing fermenters and VFA-consuming syntrophs. This study addressed this problem by developing an intermittent oxidation-reduction potential (ORP)-controlled micro-aeration system for stabilizing high OLR AD of lignocellulosic biomass. The main discovers of this study are:

- Increasing OLR resulted in proportional increases in total VFA concentration, VFA/ALK ratio that caused reduction in pH, which served as early warning indicators for AD processes.
- Rapid accumulation of acetate and reduction of methane yield were indications of methanogenesis inhibition and potential reactor failure.
- Mono-digestion of Napier grass with reactor configuration and operation of this study was limited at OLR of 5 g VS/L/day.
- Intermittent (24 h on - 24 h off) ORP-controlled micro-aeration rapidly reduced VFA concentration and recovered reactor performance from the verge of failure within 2 weeks.
- Controlling ORP (E_{Ag}) at -470 to -475 mV (i.e. +25 mV from anaerobic baseline ORP) showed best result in VFA reduction.
- The ORP-based micro-aeration provided O_2 as the limiting electron acceptor for facultative heterotrophs allowing partial aerobic oxidation of VFA while conserving crucial anaerobic niches for methanogens.

6.2 Recommendations for future study

This study, within its scope, proved the concept and opened up a novel operational strategy to operate anaerobic digesters at high OLRs that has significant economic and logistical merits for full-scale implementation of AD of lignocellulosic biomass. To

further develop this micro-aeration process control technology, further studies are recommended:

- Examine metatranscriptomic analyses of transcriptional activities during anaerobic and micro-aerobic condition to confirm the proposed pathway in this study.
- Analyze carbon isotope to identify VFA conversion and methanogenesis pathway under micro-aerobic condition.
- Analyze the effectiveness of this process in different AD reactor configurations and substrates.
- Develop mathematical models to model micro-aerated AD process with micro-oxygen transfer and utilization rate for scale up purpose.

Appendix A

Oxidation-reduction potential (ORP) process control and factors affecting ORP

Definition: Oxidation-reduction (or redox) potential of a solution is defined as the tendency of the solution to gain or lose electron (Rittmann and McCarty, 2001). A solution with positive ORP value indicates an oxidizing environment (higher tendency to gain electron, e.g. ½ $O_2 + 2H^+ + 2e^- = H_2O$), whereas a solution with negative ORP value is a reducing environment (higher tendency to lose electron, e.g. $H_2 = 2H^+ + 2e^-$). The oxidation and reduction half-reaction always occurs simultaneously to have a sum reaction, e.g. ½ $O_2 + H_2 = H_2O$)

Measurement: The ORP of a solution was originally measured with standard hydrogen electrode (SHE) as reference half-cell and the value is reported as E_h (mV). However, the application of ORP electrode with Ag/AgCl reference electrode (E_{Ag}) has gained popularity due to ease of operation and maintenance. The correlation between these 2 types of electrode according to the manufacture (Cole Parmer, USA) is: $E_{Ag} = E_h + 200$ mV.

Factors affecting ORP: According to Nernst equation, value of ORP measured with SHE (E_h) and standard redox potential (E^o) is correlated. Also, the equation also shows how increasing temperature and pH resulted in reduction in E_h, which were illustrated by results from experiment in Figure A.1 and A.2.

For reaction: $aA + n\,e^- + h\,H^+ = bB$

$$E_h = E^o - \frac{RT}{nF}\left(\log\frac{[B]^b}{[A]^a} + h\,pH\right)$$

Where, E_h is ORP with SHE; E^o is standard redox potential, R is gas constant 8.314 J/mol.K; T is temperature (K); n is number of electron transferred; F is Faraday constant 96.485 kJ/mol; h is number of proton transferred.

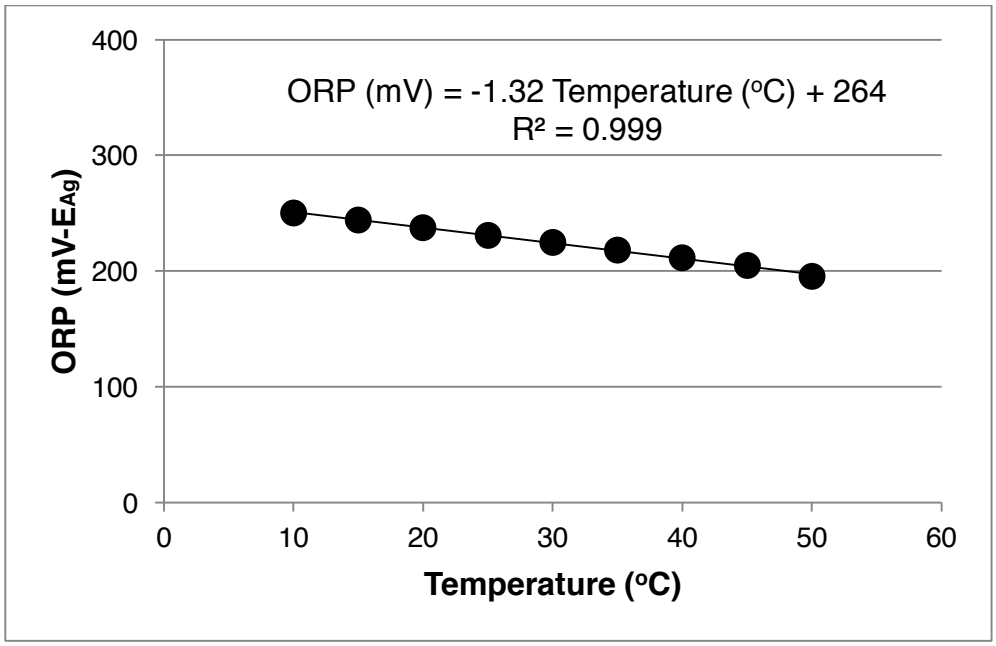

Figure A.1 Effect of temperature on ORP

Note: Data from of Zobell solution from the manufacturer (Fisher Sci., USA)

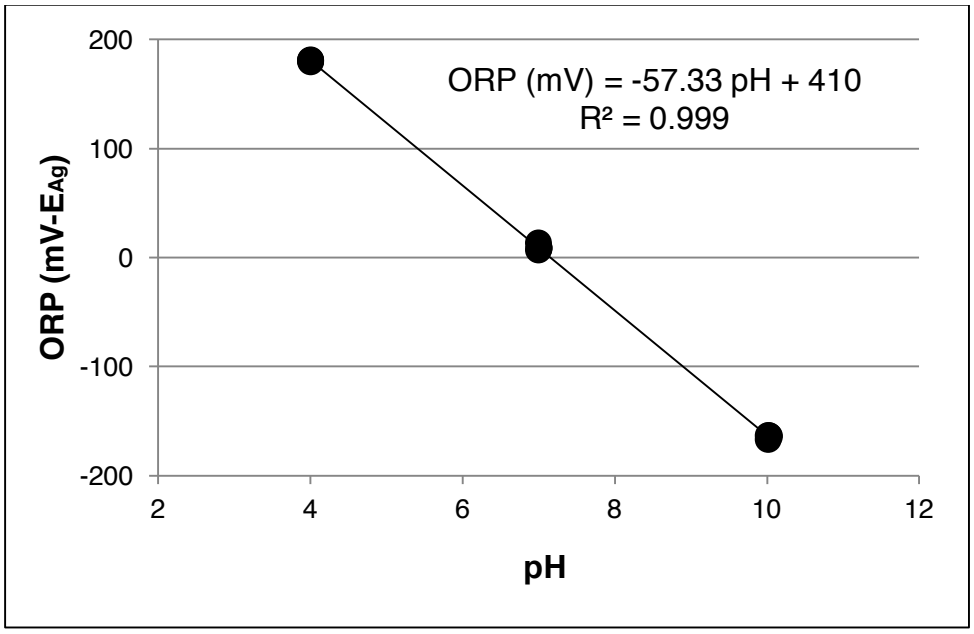

Figure A.2 Effect of pH on ORP.

Note: Measured in triplicate using pH and ORP probes on pH buffer solutions of pH 4, 7, and 10 at 25°C (Fisher Sci., US).

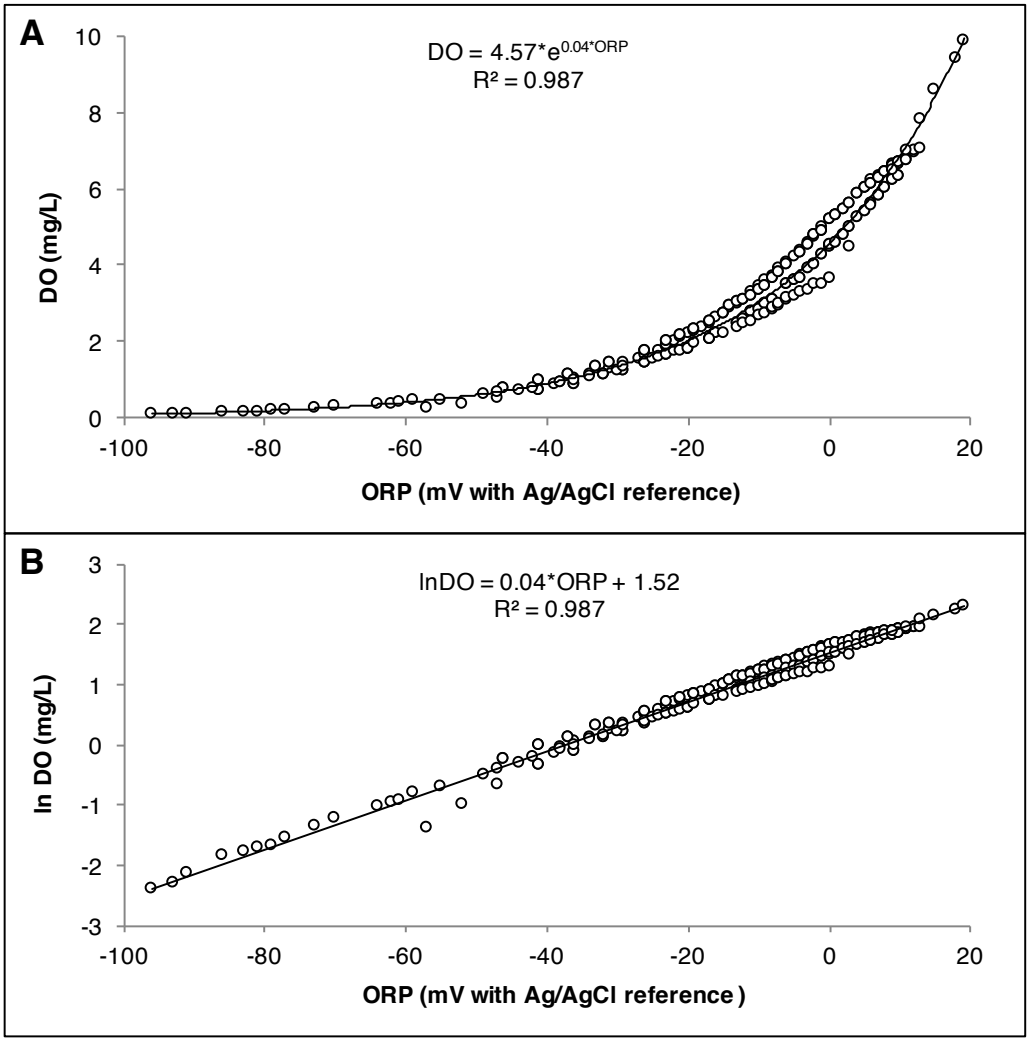

Figure A.3 Correlation between ORP with dissolved oxygen (DO) (A) and ORP with natural logarithm of DO (lnDO) (B)

Note: Experiment was conducted in triplicate at standard conditions (1 atm, 25^{o}C, pH 7.0) in effluent of reactor operated at OLR 5.0 g VS/L/day from stable period (day 80 to 83). The effluent was filtered through GF/C paper to eliminate microbial oxygen consumption. The experiment was conducted in 120 mL gas-tight bottle filled with 100 mL of prepared effluent. The bottle was flushed with N_2 gas for 5 min to created anaerobic condition (DO reached lower limit of 0.1 mg/L). Air was then slowly injected into the bottle using a glass syringe while ORP and DO probes were used to monitor and record values every 5 s.

Appendix B

Supporting results

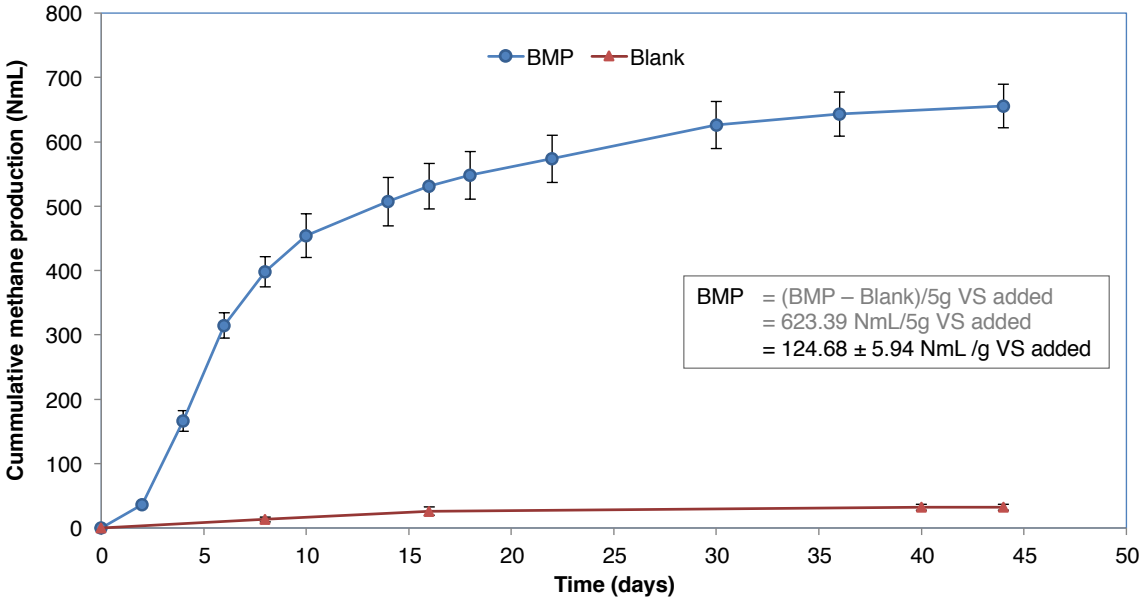

Figure B.1 Biomethane potential (BMP) test of Napier grass as substrate.

Note: Method followed (Angelidaki et al., 2009) with substrate to inoculum (S/I) ratio of 1:1 with 5 g VS added. Data shown as mean with error bars represent standard deviation from 3 replicates (error bars could be covered by markers at some points). VS removal = 78.52 ± 1.26 %; total VFA= 441 ± 13 mg/L as HAc; total ALK = 2828 ± 18 mg/L as $CaCO_3$; VFA/ALK ratio = 0.16 ± 0.00.

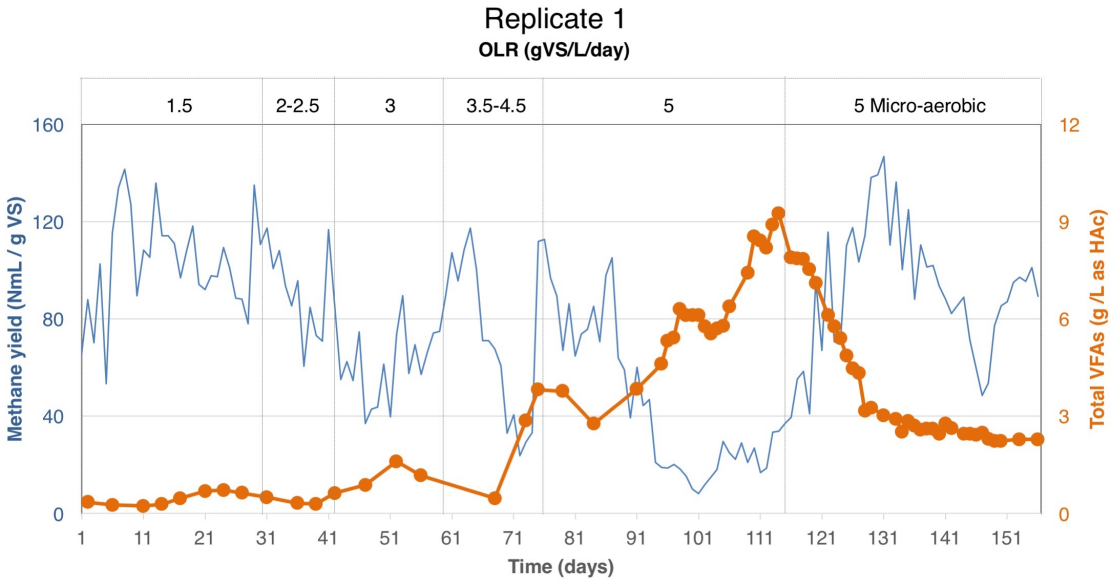

Figure B.2 Long-term performance of the first AD reactor at different OLRs.

Note: Day 77 is equivalent to day 1 in Figure 4.2

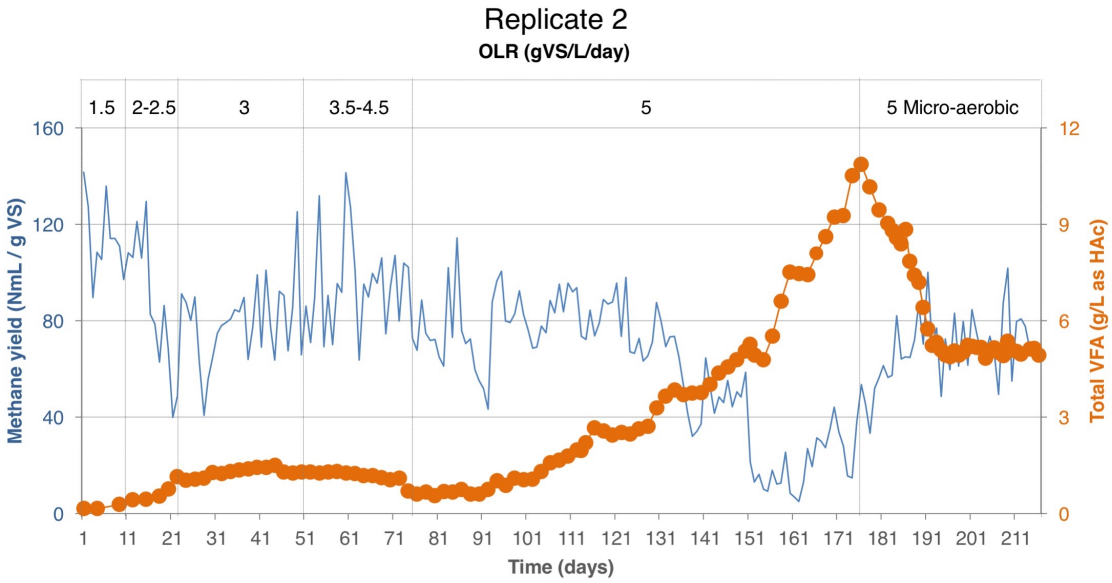

Figure B.3 Long-term performance of the second AD reactor at different OLRs.

Note: Day 137 is equivalent to day 1 in Figure 4.3

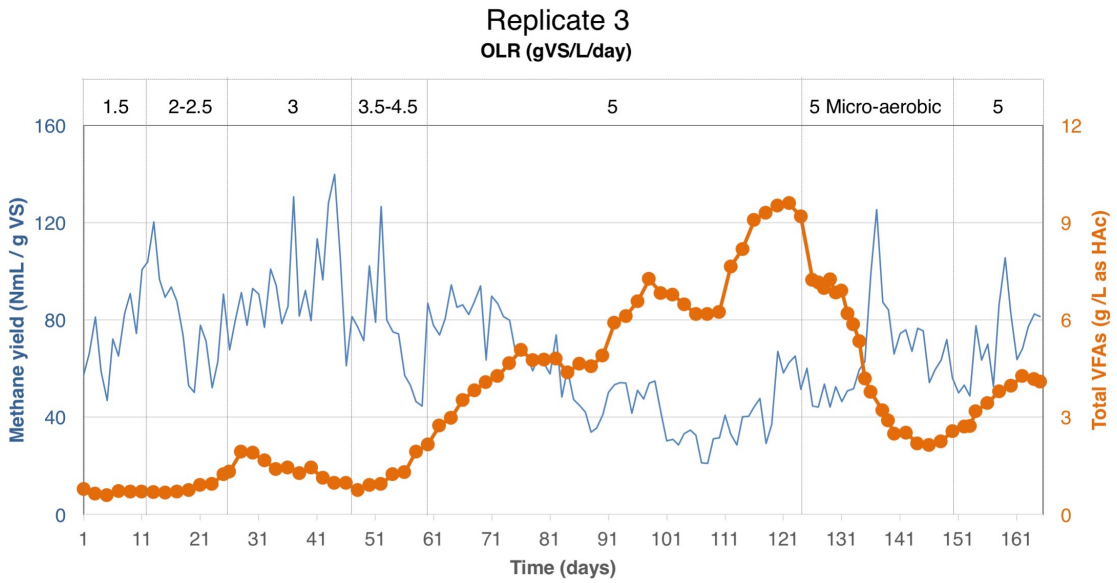

Figure B.4 Long-term performance of the third AD reactor at different OLRs.

Note: Day 83 is equivalent to day 1 in Figure 4.4

Table B.1 Taxonomic classification, relative abundance, and potential functions of bacterial and archaeal OTUs in reactor under anaerobic and micro-aerobic condition

		Relative abundance (%)			
OTUs	Genus	An-aerobic	Micro-aerobic	Potential functions	Reference
Bacteria Phylum: *Bacteroidetes*					
1, 16, 51	*Proteiniphilum*	21.2	44.3	Acidogenesis. Facultative. Ferment pyruvate, high lignin, humus and protein media producing acetate and CO_2[a]. Have	(Chen and Dong, 2005; Z. Guo et al., 2015; Hahnke et al., 2015)

				genes for cellulases, H_2 and VFA productions.[b]	
3	*Ruminofilibacter*	4.0	5.3	Hydrolysis. Facultative. Xylan degrader producing sugars and H_2.[a]	(Kröber et al., 2009)
10	vadinBC27 wastewater sludge group	0.5	2.1	Acidogenesis. Anaerobic. Glucose fermenters producing acetate, propionate, suiccinate, H_2, and CO_2.[b]	(Ni et al., 2015; Su et al., 2014)
14, 28	*Rikenellaceae* RC9 gut group	5.0	5.2	Acidogenesis. Anaerobic. Glucose fermenters producing acetate, propionate, suiccinate, H_2, and CO_2.[b]	(Ni et al., 2015; Su et al., 2014)
2	*Bacteroides*	8.0	1.4	Hydrolysis. Facultative. Hemicellulose degraders grow on xylan, xylose, sometime cellobiose producing sugars and H_2.[a]	(Centanni et al., 2017; Loesche, 1969)
26	*Phocaeicola*	0.1	3.5	Hydrolysis. Anaerobic.[a]	(Al Masalma et al., 2009)
Phylum: *Proteobacteria*					
12	*Vibrio*	0.7	1.5	Acidogenesis. Anaerobic. Hydrogen	(Iannotti et al., 1973)

				utilizing bacteria, producing acetate and succinate.[a]	
34	*Acinetobacter*	1.2	0.2	Acidogenesis. Facultative. Ferment phosphate substrate producing organic acids.[a]	(Gulati et al., 2010)
45	Unclassified *Rhodocyclaceae*	2.0	0.1	Hydrolysis and acidogenesis. Facultative. Methylotrophic bacteria using ethanol, methanol, methylamine, and other organic acids.[b]	(Smalley et al., 2015)
48	*Vulgatibacter*	2.9	0.1	Hydrolysis. Aerobic bacteria degrade organic matter in soil.[a]	(Yamamoto et al., 2014)
Phylum: *Spirochaetae*					
63	*Brevinema*	1.7	0.1	Hydrolysis. Facultative. Have esterase and phosphatases enzymes.[a]	(Bergey, 2010)
Phylum: *Firmicutes*					
4	*Mobilitalea*	2.1	0.9	Hydrolysis. Facultative. Cellulose, hemicellulose degraders producing ethanol, formate, H_2, CO_2.[a]	(Podosokorskaya et al., 2014)

38	*Lachnoclostridium*	1.9	0.1	Hydrolysis. Facultative. Cellulose and plant cell wall polysaccharides degrader by producing free cellulose-degrading enzymes.[a]	(Ravachol et al., 2015)
80	Unclassified *Gracilibacteraceae*	2.6	0.2	Acidogenesis. Anaerobic. Ferment glucose producing acetate, lactate, ethanol.[a]	(Lee et al., 2006)
21	*Sedimentibacter*	0.5	3.2	Acidogenesis. Anaerobic. Pyruvate and amino acids fermenter producing acetate and butyrate. No H_2 produced.[a]	(Breitenstein et al., 2002)
42	*Fonticella*	1.6	0.1	Hydrolysis and acidogenesis. Anaerobic. Cellobiose, starch, glucose fermenter producing formate, acetate, ethanol, and CO_2.[a]	(Fraj et al., 2013)
9, 19	*Ruminiclostridium*	4.5	1.3	Hydrolysis. Anaerobic. Cellulose and plant cell wall polysaccharides degrader with cellulosome enzyme complex.[a]	(Ravachol et al., 2015)
44	Unclassified *Ruminococcaceae*	1.1	0.2	Hydrolysis. Anaerobic. Cellulose and	(Ravachol et al., 2015)

					plant cell wall polysaccharides degrader with cellulosome enzyme complex. Mammal gut bacteria.[a]	
6, 8	*Christensenellaceae* group	R-7	5.1	4.7	Hydrolysis and acidogenesis. Anaerobic. Rumen bacteria ferment glucose producing acetate, butyrate.[b]	(Morotomi et al., 2011)
93	Unclassified *Acidaminococcaceae*		1.4	0.9	Acidogenesis. Anaerobic. Amino acid fermenters producing acetate, butyrate, sometimes succinate and propionate.[b]	(Marchandin et al., 2010)
*Syntrophs**	-		0.43*	0.24	Acetogenesis. Anaerobic. Decompose a variety of organic materials (usually fatty acids, alcohols and aromatic compounds) to produce acetate and H_2, through thermodynamically endergonic reactions at standard conditions.	(Narihiro et al., 2015)
Archaea						
Phylum: *Euryarchaeota*						

2, 5, 15	*Methanosarcina*	73.2	73.7	Mixotrophic (aceticlastic, hydrogenotrophic, methanotrophic) methanogenesis.[a] Some strains can produce antioxidative enzymes for oxidant tolerance capability	(Demirel and Scherer, 2008; Horne and Lessner, 2013)
3, 8	*Methanobacterium*	14.8	10.1	Hydrogenotrophic methanogenesis.[a] Some strains can produce antioxidative enzymes for oxidant tolerance capability	(Demirel and Scherer, 2008)
16, 18	*Methanospirillum*	4.8	10.4	Hydrogenotrophic methanogenesis.[a]	(Demirel and Scherer, 2008)
Total		92.8	94.2		

Note: OTUs: operational taxonomic units. Only OTUs with a relative abundance >1% at the genus level in at least one of the samples are included, except for syntrophs. *Relative abundance of sum of all syntrophs. Anaerobic and micro-aerobic biomass samples were collected on days 21 and 71, respectively, of reactor 1 at OLR of 5 g VS/L/day (Figure 4.2). Potential functions of microorganisms were obtained from references at genus ([a]) or family ([b]) level. NA: not available

Appendix C

Pictures of experimental set up

Figure C.1. Feedstock and inoculum preparation.

Note: Napier grass in plantation field at 5-month old (a); shredded and dried Napier grass (b); milled (<2mm size) processed Napier grass as feedstock for bioreactor (c); cow manure collection (d); pre-digested cow manure in inoculum reactor (e); cow manure-derived inoculum to start up bioreactor.

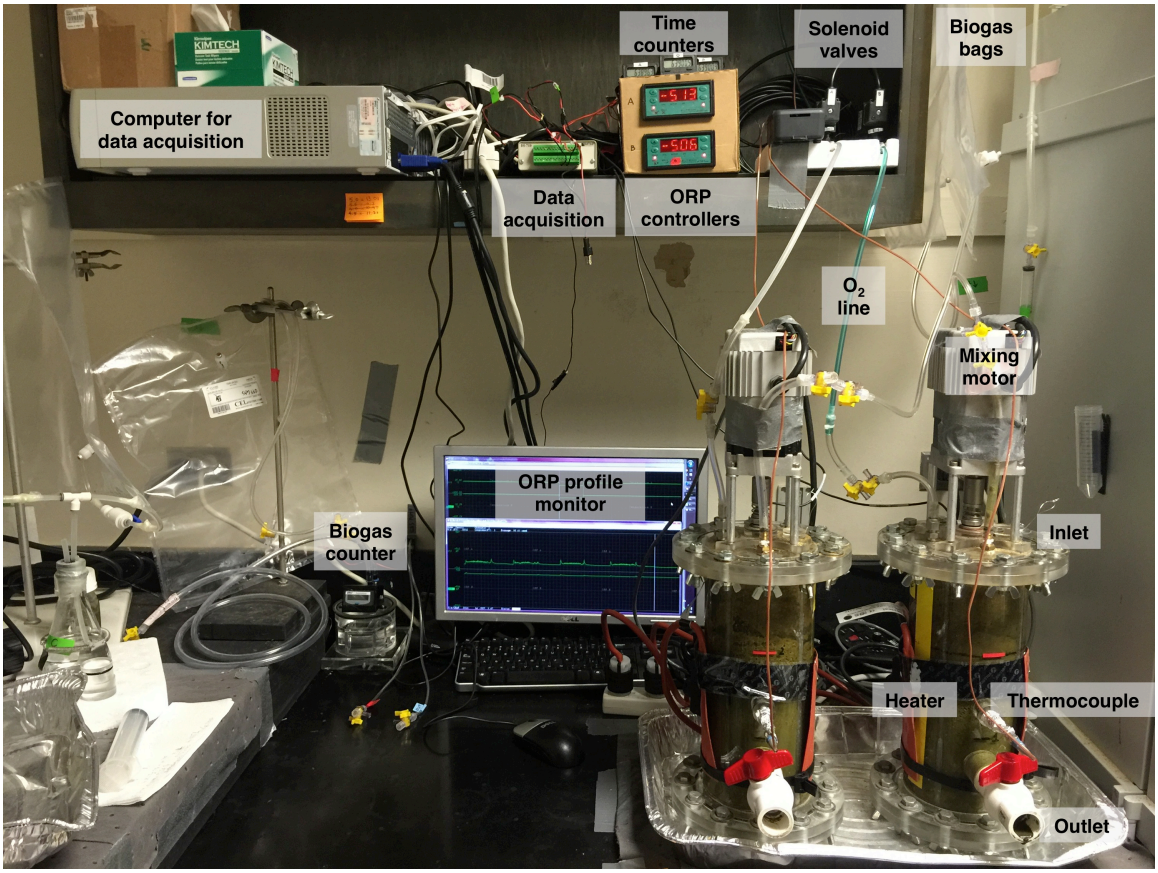

Figure C.2. Bioreactor equipped with ORP-based micro-aeration process control system.

Appendix D

List of publications and conferences

Peer-reviewed publications:

1. **Nguyen, D.**, Gadhamshetty, V., Nitayavardhana, S., & Khanal, S. K. (2015). Automatic process control in anaerobic digestion technology: A critical review. Bioresource technology, 193, 513-522.
2. Sawatdeenarunat, C., **Nguyen, D.**, Surendra, K. C., Shrestha, S., Rajendran, K., Oechsner, H., Xie, L., & Khanal, S. K. (2016). Anaerobic biorefinery: current status, challenges, and opportunities. Bioresource technology, 215, 304-313.
3. **Nguyen, D.**, Wu, Z., Shrestha, S., Lee, P.H., Raskin, L., & Khanal, S.K. (2018). High organic loading rate anaerobic digestion via bypassing syntrophic acetogenesis through intermittent micro-aeration. Environmental Science & Technology (major revision).
4. **Nguyen, D. and** Khanal, S. K. (2018). A little breath of fresh air into an anaerobic system: how microaeration facilitates anaerobic digestion process. Biotechnology Advances (submitted)**.**

Conferences

1. **Nguyen, D.** and Khanal, S.K. Student Research Symposium (SRS). 2015 (Poster), 2016 (Poster) and 2018 (Oral, 30th Annual Award Ph.D. Oral Presentation and 3 Minutes Elevator Pitch-3MEP People's Choice Award, 2018).

2. **Nguyen, D.,** Lee, P.H., Shrestha, S., Raskin, L., and Khanal, S. K. Poster. Anaerobic Digestion 2017 Conference, October 17-20, 2017, Beijing, China. (Outstanding Poster Award).

3. **Nguyen, D**. and Khanal, S.K. Poster. S-1041 Research Meeting. Aug 10-11, 2015, Wooster, OH.

References

Ahring, B.K., Sandberg, M., Angelidaki, I., 1995. Volatile fatty acids as indicators of process imbalance in anaerobic digestors. Appl. Microbiol. Biotechnol. 43, 559–565. doi:10.1007/BF00218466

Al Masalma, M., Raoult, D., Roux, V., 2009. *Phocaeicola abscessus* gen. nov., sp. nov., an anaerobic bacterium isolated from a human brain abscess sample. Int. J. Syst. Evol. Microbiol. 59, 2232–2237. doi:10.1099/ijs.0.007823-0

Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, J.L., Guwy, a. J., Kalyuzhnyi, S., Jenicek, P., Van Lier, J.B., 2009. Defining the biomethane potential (BMP) of solid organic wastes and energy crops: A proposed protocol for batch assays. Water Sci. Technol. 59, 927–934. doi:10.2166/wst.2009.040

Angle, J.C., Morin, T.H., Solden, L.M., Narrowe, A.B., Smith, G.J., Borton, M.A., Rey-Sanchez, C., Daly, R.A., Mirfenderesgi, G., Hoyt, D.W., Riley, W.J., Miller, C.S., Bohrer, G., Wrighton, K.C., 2017. Methanogenesis in oxygenated soils is a substantial fraction of wetland methane emissions. Nat. Commun. 8, 1567. doi:10.1038/s41467-017-01753-4

APHA-AWWA-WEF, 2005. Standard methods for the examination of water and wastewater. APHA-AWWA-WEF, Washington, D.C.

Ariesyady, H.D., Ito, T., Okabe, S., 2007. Functional bacterial and archaeal community structures of major trophic groups in a full-scale anaerobic sludge digester. Water Res. 41, 1554–1568. doi:10.1016/j.watres.2006.12.036

Bergey, D.H., 2010. Bergey's Manual of Systematic Bacteriology - Vol 4: Bacteroidetes, Springer-Verlag New York Inc. doi:10.1007/978-0-387-68572-4

Boe, K., Angelidaki, I., 2012. Pilot-scale application of an online VFA sensor for monitoring and control of a manure digester. Water Sci. Technol. 66, 2496–503. doi:10.2166/wst.2012.498

Boe, K., Batstone, D.J., Steyer, J.-P., Angelidaki, I., 2010. State indicators for monitoring the anaerobic digestion process. Water Res. 44, 5973–80. doi:10.1016/j.watres.2010.07.043

Botheju, D., Bakke, R., 2011. Oxygen effects in anaerobic digestion – a review. Open Waste Manag. J. 4, 1–19.

Botheju, D., Lie, B., Bakke, R., 2010. Oxygen effects in anaerobic digestion - I. Model. Identif. Control 31, 55–65. doi:10.4173/mic.2010.2.2

Breitenstein, A., Wiegel, J., Haertig, C., Weiss, N., Andreesen, J.R., Lechner, and U., 2002. Reclassification of Clostridium hydroxybenzoicum as Sedimentibacter hydroxybenzoicus gen. nov., comb. nov., and description of Sedimentibacter saalensis sp. nov. Int. J. Syst. Bacteriol. 52, 801–807. doi:10.1099/ijs.0.01998-0.Abbreviations

Brioukhanov, A.L., Netrusov, A.I., Eggen, R.I.L., 2006. The catalase and superoxide dismutase genes are transcriptionally up-regulated upon oxidative stress in the strictly anaerobic archaeon Methanosarcina barkeri. Microbiology 152, 1671–1677. doi:10.1099/mic.0.28542-0

Brown, D., Li, Y., 2013. Solid state anaerobic co-digestion of yard waste and food waste for biogas production. Bioresour. Technol. 127, 275–80. doi:10.1016/j.biortech.2012.09.081

Camiloti, P.R., Mockaitis, G., Domingues Rodrigues, J.A., Rissato Zamariolli Damianovic, M.H., Foresti, E., Zaiat, M., 2014. Innovative anaerobic bioreactor with fixed-structured bed (ABFSB) for simultaneous sulfate reduction and organic matter removal. J. Chem. Technol. Biotechnol. 89, 1044–1050. doi:10.1002/jctb.4199

Caporaso, J.G., Lauber, C.L., Walters, W. a, Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J. a, Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 6, 1621–1624.

doi:10.1038/ismej.2012.8

Centanni, M., Hutchison, J.C., Carnachan, S.M., Daines, A.M., Kelly, W.J., Tannock, G.W., Sims, I.M., 2017. Differential growth of bowel commensal *Bacteroides* species on plant xylans of differing structural complexity. Carbohydr. Polym. 157, 1374–1382. doi:10.1016/j.carbpol.2016.11.017

Chang, Y., Chang, Y., Hung, C., Lee, J., Liao, H., 2014. Microbial community analysis of anaerobic bio-corrosion in different ORP profiles. Int. Biodeterior. Biodegradation 95, 93–101. doi:10.1016/j.ibiod.2014.04.008

Chen, S., Dong, X., 2005. *Proteiniphilum acetatigenes* gen. nov., sp. nov., from a UASB reactor treating brewery wastewater. Int. J. Syst. Evol. Microbiol. 55, 2257–2261. doi:10.1099/ijs.0.63807-0

Chen, Y., Cheng, J.J., Creamer, K.S., 2008. Inhibition of anaerobic digestion process: a review. Bioresour. Technol. 99, 4044–64. doi:10.1016/j.biortech.2007.01.057

De Vrieze, J., Hennebel, T., Boon, N., Verstraete, W., 2012. Methanosarcina: The rediscovered methanogen for heavy duty biomethanation. Bioresour. Technol. 112, 1–9. doi:10.1016/j.biortech.2012.02.079

Demirel, B., Scherer, P., 2008. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review. Rev. Environ. Sci. Bio/Technology 7, 173–190. doi:10.1007/s11157-008-9131-1

Diak, J., Örmeci, B., Kennedy, K.J., 2013. Effect of micro-aeration on anaerobic digestion of primary sludge under septic tank conditions. Bioprocess Biosyst. Eng. 36, 417–424. doi:10.1007/s00449-012-0798-x

Díaz, I., Donoso-Bravo, A., Fdz-Polanco, M., 2011a. Effect of microaerobic conditions on the degradation kinetics of cellulose. Bioresour. Technol. 102, 10139–10142. doi:10.1016/j.biortech.2011.07.096

Díaz, I., Lopes, a. C., Pérez, S.I., Fdz-Polanco, M., 2010. Performance evaluation of oxygen, air and nitrate for the microaerobic removal of hydrogen sulphide in biogas

from sludge digestion. Bioresour. Technol. 101, 7724–7730. doi:10.1016/j.biortech.2010.04.062

Díaz, I., Pérez, S.I., Ferrero, E.M., Fdz-Polanco, M., 2011b. Effect of oxygen dosing point and mixing on the microaerobic removal of hydrogen sulphide in sludge digesters. Bioresour. Technol. 102, 3768–3775. doi:10.1016/j.biortech.2010.12.016

Díaz, I., Ramos, I., Fdz-Polanco, M., 2015. Economic analysis of microaerobic removal of H 2 S from biogas in full-scale sludge digesters. Bioresour. Technol. 192, 280–286. doi:10.1016/j.biortech.2015.05.048

Dolfing, J., 2015. Protocols for Calculating Reaction Kinetics and Thermodynamics, in: Hydrocarbon and Lipid Microbiology Protocols. Springer, pp. 1–29. doi:10.1007/8623

Dolfing, J., 2014. Thermodynamic constraints on syntrophic acetate oxidation. Appl. Environ. Microbiol. 80, 1539–1541. doi:10.1128/AEM.03312-13

Dong, F., Zhao, Q.B., Li, W.W., Sheng, G.P., Zhao, J.B., Tang, Y., Yu, H.Q., Kubota, K., Li, Y.Y., Harada, H., 2011. Novel online monitoring and alert system for anaerobic digestion reactors. Environ. Sci. Technol. 45, 9093–9100. doi:10.1021/es202245f

Dykstra, C.M., Pavlostathis, S.G., 2017. Methanogenic biocathode microbial community development and the role of bacteria. Environ. Sci. Technol. 51, 5306–5316.

EBA, 2018. Biogas Production in Europe: Biogas Report 2018.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200. doi:10.1093/bioinformatics/btr381

EPA, 2018. AgSTAR database of livestock digesters.

Ezraty, B., Gennaris, A., Barras, F., Collet, J.-F., 2017. Oxidative stress, protein damage and repair in bacteria. Nat. Rev. Microbiol. doi:10.1038/nrmicro.2017.26

Faithfull, N.T., 2002. Methods in Agricultural Chemical Analysis: A Practical Handbook.

CABI Publishing, New York, NY, USA.

Fdz.-Polanco, M., Díaz, I., Pérez, S.I., Lopes, A.C., Fdz.-Polanco, F., 2009. Hydrogen sulphide removal in the anaerobic digestion of sludge by micro-aerobic processes: Pilot plant experience. Water Sci. Technol. 60, 3045–3050. doi:10.2166/wst.2009.738

Feitkenhauer, H., Von, S.J., Meyer, U., 2002. On-line titration of volatile fatty acids for the process control of anaerobic digestion plants. Water Res. 36, 212–8.

Fernández-Sandoval, M.T., Galíndez-Mayer, J., Moss-Acosta, C.L., Gosset, G., Martinez, A., 2017. Volumetric oxygen transfer coefficient as a means of improving volumetric ethanol productivity and a criterion for scaling up ethanol production with Escherichia coli. J. Chem. Technol. & Biotechnol. 92, 981–989. doi:10.1002/jctb.5087

Fish, J.A., Chai, B., Wang, Q., Sun, Y., Brown, C.T., Tiedje, J.M., Cole, J.R., 2013. FunGene: The functional gene pipeline and repository. Front. Microbiol. 4, 1–14. doi:10.3389/fmicb.2013.00291

Forster, A.H., Gescher, J., 2014. Metabolic engineering of *Escherichia coli* for production of mixed-acid fermentation end products. Front. Bioeng. Biotechnol. 2, 1–12. doi:10.3389/fbioe.2014.00016

Fraj, B., Hania, W. Ben, Postec, A., Hamdi, M., Ollivier, B., Fardeau, M.L., 2013. Fonticella tunisiensis gen. nov., sp. nov., isolated from a hot spring. Int. J. Syst. Evol. Microbiol. 63, 1947–1950. doi:10.1099/ijs.0.041947-0

Fu, H., Yuan, J., Gao, H., 2015. Microbial oxidative stress response: Novel insights from environmental facultative anaerobic bacteria. Arch. Biochem. Biophys. 584, 28–35. doi:10.1016/j.abb.2015.08.012

Fu, S.-F., Wang, F., Yuan, X.-Z., Yang, Z.-M., Luo, S.-J., Wang, C.-S., Guo, R.-B., 2014. The thermophilic (55°C) microaerobic pretreatment of corn straw for anaerobic digestion. Bioresour. Technol. 175C, 203–208. doi:10.1016/j.biortech.2014.10.072

Fu, S.F., Wang, F., Shi, X.S., Guo, R.B., 2016. Impacts of microaeration on the anaerobic digestion of corn straw and the microbial community structure. Chem. Eng. J. 287, 523–528. doi:10.1016/j.cej.2015.11.070

Garcia-ochoa, F., Gomez, E., 2009. Bioreactor scale-up and oxygen transfer rate in microbial processes : An overview. Biotechnol. Adv. 27, 153–176. doi:10.1016/j.biotechadv.2008.10.006

Girotto, F., Peng, W., Rafieenia, R., Cossu, R., 2016. Effect of Aeration Applied During Different Phases of Anaerobic Digestion. Waste and Biomass Valorization. doi:10.1007/s12649-016-9785-9

Goux, X., Calusinska, M., Fossepre, M., Benizri, E., Delfosse, P., 2016. Start-up phase of an anaerobic full-scale farm reactor - Appearance of mesophilic anaerobic conditions and establishment of the methanogenic microbial community. Bioresour. Technol. 212, 217–226. doi:10.1016/j.biortech.2016.04.040

Goux, X., Calusinska, M., Lemaigre, S., Marynowska, M., Klocke, M., Udelhoven, T., Benizri, E., Delfosse, P., 2015. Microbial community dynamics in replicate anaerobic digesters exposed sequentially to increasing organic loading rate, acidosis, and process recovery. Biotechnol. Biofuels 8, 1–18. doi:10.1186/s13068-015-0309-9

Gulati, A., Sharma, N., Vyas, P., Sood, S., Rahi, P., Pathania, V., Prasad, R., 2010. Organic acid production and plant growth promotion as a function of phosphate solubilization by *Acinetobacter rhizosphaerae* strain BIHB 723 isolated from the cold deserts of the trans-Himalayas. Arch. Microbiol. 192, 975–983. doi:10.1007/s00203-010-0615-3

Guo, J., Peng, Y., Ni, B.-J., Han, X., Fan, L., Yuan, Z., 2015. Dissecting microbial community structure and methane-producing pathways of a full-scale anaerobic reactor digesting activated sludge from wastewater treatment by metagenomic sequencing. Microb. Cell Fact. 14, 33. doi:10.1186/s12934-015-0218-4

Guo, Z., Zhou, A., Yang, C., Liang, B., Sangeetha, T., He, Z., Wang, L., Cai, W., Wang, A., Liu, W., 2015. Enhanced short chain fatty acids production from waste activated

sludge conditioning with typical agricultural residues: carbon source composition regulates community functions. Biotechnol. Biofuels 8, 1–14. doi:10.1186/s13068-015-0369-x

Hahnke, S., Maus, I., Wibberg, D., Tomazetto, G., Pühler, A., Klocke, M., Schlüter, A., 2015. Complete genome sequence of the novel *Porphyromonadaceae bacterium* strain ING2-E5B isolated from a mesophilic lab-scale biogas reactor. J. Biotechnol. 193, 34–36. doi:10.1016/j.jbiotec.2014.11.010

Hanreich, A., Schimpf, U., Zakrzewski, M., Schlüter, A., Benndorf, D., Heyer, R., Rapp, E., Pühler, A., Reichl, U., Klocke, M., 2013. Metagenome and metaproteome analyses of microbial communities in mesophilic biogas-producing anaerobic batch fermentations indicate concerted plant carbohydrate degradation. Syst. Appl. Microbiol. 36, 330–338. doi:10.1016/j.syapm.2013.03.006

Hansson, M., Nordberg, a, Sundh, I., Mathisen, B., 2002. Early warning of disturbances in a laboratory-scale MSW biogas process. Water Sci. Technol. 45, 255–60.

Hasegawa, S., Shiota, N., Katsura, K., Akashi, A., 2000. Solubilization of organic sludge by thermophilic aerobic bacteria as a pretreatment for anaerobic digestion 163–169.

Hattorii, S., 2008. Syntrophic Acetate-Oxidizing Microbes in Methanogenic Environments. Microbes Environ. 23, 118–127.

Hill, D.T., Cobb, S.A., Bolte, J.P., 1987. Using Volatile Fatty Acid Relationships to Predict Anaerobic Digester Failure. Trans. ASAE 30, 0496–0501. doi:10.13031/2013.31977

Horne, A.J., Lessner, D.J., 2013. Assessment of the oxidant tolerance of Methanosarcina acetivorans. FEMS Microbiol. Lett. 343, 13–19. doi:10.1111/1574-6968.12115

Iannotti, E.L., Kafkewit, D., Wolin, M.J., Bryant, M.P., 1973. Glucose fermentation products of *Ruminococcus albus* grown in continuous culture with *Vibrio succinogenes* - changes caused by interspecies transfer of H2. J. Bacteriol. 114, 1231–1240.

Imlay, J. a, 2013. The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. Nat. Rev. Microbiol. 11, 443–54. doi:10.1038/nrmicro3032

Jagadabhi, P.S., Kaparaju, P., Rintala, J., 2010a. Effect of micro-aeration and leachate replacement on COD solubilization and VFA production during mono-digestion of grass-silage in one-stage leach-bed reactors. Bioresour. Technol. 101, 2818–24. doi:10.1016/j.biortech.2009.10.083

Jagadabhi, P.S., Kaparaju, P., Rintala, J., 2010b. Effect of micro-aeration and leachate replacement on COD solubilization and VFA production during mono-digestion of grass-silage in one-stage leach-bed reactors. Bioresour. Technol. 101, 2818–2824. doi:10.1016/j.biortech.2009.10.083

Jenicek, P., Celis, C. a., Koubova, J., Pokorna, D., 2011. Comparison of microbial activity in anaerobic and microaerobic digesters. Water Sci. Technol. 63, 2244–2249. doi:10.2166/wst.2011.579

Jenicek, P., Celis, C. a., Krayzelova, L., Anferova, N., Pokorna, D., 2014. Improving products of anaerobic sludge digestion by microaeration. Water Sci. Technol. 69, 803. doi:10.2166/wst.2013.779

Johansen, J.-E., Bakke, R., 2006. Enhancing hydrolysis with microaeration. Water Sci. Technol. 53, 43. doi:10.2166/wst.2006.234

Kafle, G.K., Kim, S.-H., Shin, B.-S., 2012. Anaerobic digestion treatment for the mixture of chinese cabbage waste juice and swine manure. J. Biosyst. Eng. 37, 58–64. doi:10.5307/JBE.2012.37.1.058

Khanal, Huang, J.-C., 2003. ORP-based oxygenation for sulfide control in anaerobic treatment of high-sulfate wastewater. Water Res. 37, 2053–62. doi:10.1016/S0043-1354(02)00618-8

Khanal, S.K., 2008. Anaerobic biotechnology for bioenergy production: principles and applications. John Wiley & Sons, Inc., Ames, Iowa.

Khanal, S.K., Huang, J.-C., 2006. Online oxygen control for sulfide oxidation in anaerobic treatment of high-sulfate wastewater. Water Environ. Res. 78, 397–408.

Khanal, S.K., Li, Y., 2016. Bioenergy: Principles and Applications. John Wiley & Sons.

Khanal, S.K., Shang, C., Huang, J.C., 2003. Use of ORP (oxidation-reduction potential) to control oxygen dosing for online sulfide oxidation in anaerobic treatment of high sulfate wastewater. Water Sci. Technol. 47, 183–9.

Kleyböcker, A., Liebrich, M., Verstraete, W., Kraume, M., Würdemann, H., 2012. Early warning indicators for process failure due to organic overloading by rapeseed oil in one-stage continuously stirred tank reactor, sewage sludge and waste digesters. Bioresour. Technol. 123, 534–541. doi:10.1016/j.biortech.2012.07.089

Krayzelova, L., Bartacek, J., Díaz, I., Jeison, D., Volcke, E.I.P., Jenicek, P., 2015. Microaeration for hydrogen sulfide removal during anaerobic treatment: a review. Rev. Environ. Sci. Biotechnol. 14, 703–725. doi:10.1007/s11157-015-9386-2

Krayzelova, L., Bartacek, J., Kolesarova, N., Jenicek, P., 2014. Microaeration for hydrogen sulfide removal in UASB reactor. Bioresour. Technol. 172, 297–302. doi:10.1016/j.biortech.2014.09.056

Kröber, M., Bekel, T., Diaz, N.N., Goesmann, A., Jaenicke, S., Krause, L., Miller, D., Runte, K.J., Viehöver, P., Pühler, A., Schlüter, A., 2009. Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing. J. Biotechnol. 142, 38–49. doi:10.1016/j.jbiotec.2009.02.010

Labib, F., Ferguson, J.F., Benjamin, M.M., Merigh, M., Ricker, N.L., 1993. Mathematical modeling of an anaerobic butyrate degrading consortia: Predicting their response to organic overloads. Environ. Sci. Technol. 27, 2673–2684. doi:10.1021/es00049a005

Lackner, S., Lindenblatt, C., Horn, H., 2012. 'Swinging ORP' as operation strategy for stable reject water treatment by nitritation–anammox in sequencing batch reactors.

Chem. Eng. J. 180, 190–196. doi:10.1016/j.cej.2011.11.043

Lahav, O., Morgan, B., 2004. Titration methodologies for monitoring of anaerobic digestion in developing countries?a review. J. Chem. Technol. Biotechnol. 79, 1331–1341. doi:10.1002/jctb.1143

Lawrence, A.W., McCarty, P.L., 1969. Kinetics of Methane Fermentation in Anaerobic Treatment. J. Water Pollut. Control Fed. 41, 1–17. doi:10.2307/25036255

Lee, Y.J., Romanek, C.S., Mills, G.L., Davis, R.C., Whitman, W.B., Wiegel, J., 2006. *Gracilibacter thermotolerans* gen. nov., sp. nov., an anaerobic, thermotolerant bacterium from a constructed wetland receiving acid sulfate water. Int. J. Syst. Evol. Microbiol. 56, 2089–2093. doi:10.1099/ijs.0.64040-0

Leng, L., Yang, P., Singh, S., Zhuang, H., Xu, L., Chen, W.H., Dolfing, J., Li, D., Zhang, Y., Zeng, H., Chu, W., Lee, P.H., 2017. A review on the bioenergetics of anaerobic microbial metabolism close to the thermodynamic limits and its implications for digestion applications. Bioresour. Technol. 247, 1095–1106. doi:10.1016/j.biortech.2017.09.103

Lim, J.W., Chen, C.-L., Ho, I.J.R., Wang, J.-Y., 2013. Study of microbial community and biodegradation efficiency for single- and two-phase anaerobic co-digestion of brown water and food waste. Bioresour. Technol. 147, 193–201. doi:10.1016/j.biortech.2013.08.038

Lim, J.W., Chiam, J.A., Wang, J.-Y., 2014. Microbial community structure reveals how microaeration improves fermentation during anaerobic co-digestion of brown water and food waste. Bioresour. Technol. 171, 132–8. doi:10.1016/j.biortech.2014.08.050

Lim, J.W., Wang, J.-Y., 2013. Enhanced hydrolysis and methane yield by applying microaeration pretreatment to the anaerobic co-digestion of brown water and food waste. Waste Manag. 33, 813–9. doi:10.1016/j.wasman.2012.11.013

Liu, C.-G., Lin, Y.-H., Bai, F.-W., 2011. Development of redox potential-controlled schemes for very-high-gravity ethanol fermentation. J. Biotechnol. 153, 42–7.

doi:10.1016/j.jbiotec.2011.03.007

Liu, C.-G., Xue, C., Lin, Y.-H., Bai, F.-W., 2013. Redox potential control and applications in microaerobic and anaerobic fermentations. Biotechnol. Adv. 31, 257–65. doi:10.1016/j.biotechadv.2012.11.005

Liu, Y., Whitman, W.B., 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann. N. Y. Acad. Sci. 1125, 171–189. doi:10.1196/annals.1419.019

Loesche, W.J., 1969. Oxygen sensitivity of various anaerobic bacteria. Appl. Microbiol. 18, 723–727.

Luton, P.E., Wayne, J.M., Sharp, R.J., Riley, P.W., 2002. The mcrA gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. Microbiology 148, 3521–3530.

Ma, Y., Domingo-Feíez, C., Ploz, B.G., Smets, B.F., 2017. Intermittent aeration suppresses nitrite-oxidizing bacteria in membrane-aerated biofilms: a model-based explanation. Environ. Sci. Technol. 51, 6146–6155. doi:10.1021/acs.est.7b00463

Madigan, M.T., Martinko, J.M., Bender, K.S., Buckley, D.H., Stahl, D.A., 2015. Brock Biology of Microorganisms, 14th ed. Pearson., Boston.

Marchandin, H., Teyssier, C., Campos, J., Jean-Pierre, H., Roger, F., Gay, B., Carlier, J.P., Jumas-Bilak, E., 2010. *Negativicoccus succinicivorans* gen. nov., sp. nov., isolated from human clinical samples, emended description of the family *Veillonellaceae* and description of *Negativicutes* classis nov., *Selenomonadales* ord. nov. Int. J. Syst. Evol. Microbiol. 60, 1271–1279. doi:10.1099/ijs.0.013102-0

Morotomi, M., Nagai, F., Watanabe, Y., 2011. Description of *Christensenella minuta* gen. nov., sp. nov., isolated from human faeces, which forms a distinct branch in the order *Clostridiales*, and proposal of *Christensenellaceae* fam. nov. Int. J. Syst. Evol. Microbiol. 62, 144–149. doi:10.1099/ijs.0.026989-0

Morris, R.L., Schmidt, T.M., 2013. Shallow breathing: bacterial life at low O2. Nat. Rev.

Microbiol. 11, 205–212. doi:10.1038/nrmicro2970

Muyzer, G., Stams, A.J.M., 2008. The ecology and biotechnology of sulphate-reducing bacteria. Nat. Rev. Microbiol. 6, 441–454. doi:10.1038/nrmicro1892

Narihiro, T., Nobu, M.K., Kim, N., Kamagata, Y., Liu, W., 2015. The nexus of syntrophy-associated microbiota in anaerobic digestion revealed by long-term enrichment and community survey. Environ. Microbiol. 17, 1707–1720.

Nghiem, L., Manassa, P., Dawson, M., 2014a. Oxidation reduction potential as a parameter to regulate micro-oxygen injection into anaerobic digester for reducing hydrogen sulphide concentration in biogas.

Nghiem, L., Manassa, P., Dawson, M., Fitzgerald, S.K., 2014b. Oxidation reduction potential as a parameter to regulate micro-oxygen injection into anaerobic digester for reducing hydrogen sulphide concentration in biogas. Bioresour. Technol. 173, 443–447. doi:10.1016/j.biortech.2014.09.052

Nguyen, D., Gadhamshetty, V., Nitayavardhana, S., Khanal, S.K., 2015. Automatic process control in anaerobic digestion technology: A critical review. Bioresour. Technol. 193, 513–522. doi:10.1016/j.biortech.2015.06.080

Nguyen, P.H.L., Kuruparan, P., Visvanathan, C., 2007. Anaerobic digestion of municipal solid waste as a treatment prior to landfill. Bioresour. Technol. 98, 380–387. doi:10.1016/j.biortech.2005.12.018

Ni, B.J., Batstone, D., Zhao, B.H., Yu, H.Q., 2015. Microbial internal storage alters the carbon transformation in dynamic anaerobic fermentation. Environ. Sci. Technol. 49, 9159–9167. doi:10.1021/acs.est.5b01855

Nielsen, H., Uellendahl, H., Ahring, B., 2007. Regulation and optimization of the biogas process: Propionate as a key parameter. Biomass and Bioenergy 31, 820–830. doi:10.1016/j.biombioe.2007.04.004

Niu, Q., Kobayashi, T., Takemura, Y., Kubota, K., Li, Y.-Y., 2015. Evaluation of functional microbial community's difference in full-scale and lab-scale anaerobic

digesters feeding with different organic solid waste: Effects of substrate and operation factors. Bioresour. Technol. 193, 110–118. doi:10.1016/j.biortech.2015.05.107

Pind, P.F., Angelidaki, I., Ahring, B.K., 2003. Dynamics of the anaerobic process: effects of volatile fatty acids. Biotechnol. Bioeng. 82, 791–801. doi:10.1002/bit.10628

Podosokorskaya, O.A., Bonch-Osmolovskaya, E.A., Beskorovaynyy, A. V., Toshchakov, S. V., Kolganova, T. V., Kublanov, I. V., 2014. *Mobilitalea sibirica* gen. nov., sp. nov., a halotolerant polysaccharide-degrading bacterium. Int. J. Syst. Evol. Microbiol. 64, 2657–2661. doi:10.1099/ijs.0.057109-0

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., Glöckner, F.O., 2007. SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35, 7188–7196. doi:10.1093/nar/gkm864

Ragauskas, A.J., 2006. The Path Forward for Biofuels and Biomaterials. Science (80-.). 311, 484–489. doi:10.1126/science.1114736

Ramos, I., Fdz-Polanco, M., 2013. The potential of oxygen to improve the stability of anaerobic reactors during unbalanced conditions: Results from a pilot-scale digester treating sewage sludge. Bioresour. Technol. 140, 80–85. doi:10.1016/j.biortech.2013.04.066

Ramos, I., Perez, R., Fdz-Polanco, M., 2014. The headspace of microaerobic reactors: Sulphide-oxidising population and the impact of cleaning on the efficiency of biogas desulphurisation. Bioresour. Technol. 158, 63–73. doi:10.1016/j.biortech.2014.02.001

Ramos, I., Pérez, R., Fdz-Polanco, M., 2013. Microaerobic desulphurisation unit: A new biological system for the removal of H2S from biogas. Bioresour. Technol. 142, 633–640. doi:10.1016/j.biortech.2013.05.084

Ravachol, J., Borne, R., Meynial-Salles, I., Soucaille, P., Pagès, S., Tardif, C., Fierobe,

H.-P., 2015. Combining free and aggregated cellulolytic systems in the cellulosome-producing bacterium *Ruminiclostridium cellulolyticum*. Biotechnol. Biofuels 8, 1–14. doi:10.1186/s13068-015-0301-4

Rittmann, B.E., McCarty, P.L., 2001. Environmental biotechnology: principles and applications. McGrawHill, New York.

Sawatdeenarunat, C., Nam, H., Adhikari, S., Sung, S., Khanal, S.K., 2018. Decentralized biorefinery for lignocellulosic biomass: Integrating anaerobic digestion with thermochemical conversion. Bioresour. Technol. 250, 140–147. doi:10.1016/j.biortech.2017.11.020

Sawatdeenarunat, C., Nguyen, D., Surendra, K.C., Shrestha, S., Rajendran, K., Oechsner, H., Xie, L., Khanal, S.K., 2016. Anaerobic biorefinery: Current status, challenges, and opportunities. Bioresour. Technol. 215, 304–313. doi:10.1016/j.biortech.2016.03.074

Sawatdeenarunat, C., Sung, S., Khanal, S.K., 2017. Enhanced volatile fatty acids production during anaerobic digestion of lignocellulosic biomass via micro-oxygenation. Bioresour. Technol. 237, 139–145. doi:10.1016/j.biortech.2017.02.029

Sawatdeenarunat, C., Surendra, K.C., Takara, D., Oechsner, H., Kumar Khanal, S., 2015. Anaerobic digestion of lignocellulosic biomass: Challenges and opportunities. Bioresour. Technol. 178, 178–186. doi:10.1016/j.biortech.2014.09.103

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541. doi:10.1128/AEM.01541-09

Shalel-Levanon, S., San, K.Y., Bennett, G.N., 2005. Effect of oxygen, and ArcA and FNR regulators on the expression of genes related to the electron transfer chain and the TCA cycle in *Escherichia coli*. Metab. Eng. 7, 364–374.

doi:10.1016/j.ymben.2005.07.001

Sheets, J.P., Ge, X., Li, Y., 2015. Effect of limited air exposure and comparative performance between thermophilic and mesophilic solid-state anaerobic digestion of switchgrass 180, 296–303.

Shi, Y., Hu, S., Lou, J., Lu, P., Keller, J., Yuan, Z., 2013. Nitrogen removal from wastewater by coupling anammox and methane-dependent denitrification in a membrane biofilm reactor. Environ. Sci. Technol. 47, 11577–11583. doi:10.1021/es402775z

Shrestha, S., Fonoll, X., Khanal, S.K., Raskin, L., 2017. Biological strategies for enhanced hydrolysis of lignocellulosic biomass during anaerobic digestion: Current status and future perspectives. Bioresour. Technol. 245, 1245–1257. doi:10.1016/j.biortech.2017.08.089

Smalley, N.E., Taipale, S., De Marco, P., Doronina, N. V., Kyrpides, N., Shapiro, N., Woyke, T., Kalyuzhnaya, M.G., 2015. Functional and genomic diversity of methylotrophic *Rhodocyclaceae*: Description of *Methyloversatilis discipulorum* sp. nov. Int. J. Syst. Evol. Microbiol. 65, 2227–2233. doi:10.1099/ijs.0.000190

Stams, A.J.M., Plugge, C.M., 2009. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat. Rev. Microbiol. 7, 568–577. doi:10.1038/nrmicro2166

Stewart, P.S., Franklin, M.J., 2008. Physiological heterogeneity in biofilms. Nat. Rev. Microbiol. 6, 199–210. doi:10.1038/nrmicro1838

Steyer, J.P., Bernard, O., Batstone, D.J., Angelidaki, I., 2006. Lessons learnt from 15 years of ICA in anaerobic digesters. Water Sci. Technol. 53, 25–33. doi:10.2166/wst.2006.107

Su, X.L., Tian, Q., Zhang, J., Yuan, X.Z., Shi, X.S., Guo, R.B., Qiu, Y.L., 2014. *Acetobacteroides hydrogenigenes* gen. nov., sp. nov., an anaerobic hydrogen-producing bacterium in the family *Rikenellaceae* isolated from a reed swamp. Int. J.

Syst. Evol. Microbiol. 64, 2986–2991. doi:10.1099/ijs.0.063917-0

Sundberg, C., Al-Soud, W.A., Larsson, M., Alm, E., Yekta, S.S., Svensson, B.H., Sørensen, S.J., Karlsson, A., 2013. 454 Pyrosequencing Analyses of Bacterial and Archaeal Richness in 21 Full-Scale Biogas Digesters. FEMS Microbiol. Ecol. 85, 612–626. doi:10.1111/1574-6941.12148

Surendra, K.C., Khanal, S.K., 2014. Effects of crop maturity and size reduction on digestibility and methane yield of dedicated energy crop. Bioresour. Technol. 178, 187–193. doi:10.1016/j.biortech.2014.09.055

Surendra, K.C., Sawatdeenarunat, C., Shrestha, S., Sung, S., Khanal, S.K., 2015. Anaerobic digestion-based biorefinery for bioenergy and biobased products. Ind. Biotechnol. 11, 103–112. doi:10.1089/ind.2015.0001

Takara, D., Khanal, S.K., 2015. Characterizing compositional changes of Napier grass at different stages of growth for biofuel and biobased products potential. Bioresour. Technol. 188, 103–108. doi:10.1016/j.biortech.2015.01.114

Tang, Y., Shigematsu, T., Ikbal, Morimura, S., Kida, K., 2004. The effects of micro-aeration on the phylogenetic diversity of microorganisms in a thermophilic anaerobic municipal solid-waste digester. Water Res. 38, 2537–50. doi:10.1016/j.watres.2004.03.012

Thauer, R.K., Jungermann, K., Decker, K., 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41, 100–180. doi:10.1073/pnas.0803850105

Treu, L., Campanaro, S., Kougias, P.G., Zhu, X., Angelidaki, I., 2016. Untangling the Effect of Fatty Acid Addition at Species Level Revealed Different Transcriptional Responses of the Biogas Microbial Community Members. Environ. Sci. Technol. 50, 6079–6090. doi:10.1021/acs.est.6b00296

van der Zee, F.P., Villaverde, S., Garc??a, P.A., Fdz.-Polanco, F., 2007. Sulfide removal by moderate oxygenation of anaerobic sludge environments. Bioresour. Technol. 98, 518–524. doi:10.1016/j.biortech.2006.02.011

Vanwonterghem, I., Jensen, P.D., Ho, D.P., Batstone, D.J., Tyson, G.W., 2014. Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. Curr. Opin. Biotechnol. 27, 55–64. doi:10.1016/j.copbio.2013.11.004

Wagner, A.O., Reitschuler, C., Illmer, P., 2014. Effect of different acetate:propionate ratios on the methanogenic community during thermophilic anaerobic digestion in batch experiments. Biochem. Eng. J. 90, 154–161. doi:10.1016/j.bej.2014.05.014

Wang, H., Fotidis, I.A., Angelidaki, I., 2015. Ammonia effect on hydrogenotrophic methanogens and syntrophic acetate-oxidizing bacteria. FEMS Microbiol. Ecol. 91, 1–8. doi:10.1093/femsec/fiv130

Wang, L., Zhou, Q., Li, F.T., 2006. Avoiding propionic acid accumulation in the anaerobic process for biohydrogen production. Biomass and Bioenergy 30, 177–182. doi:10.1016/j.biombioe.2005.11.010

Wang, L.H., Wang, Q., Cai, W., Sun, X., 2012. Influence of mixing proportion on the solid-state anaerobic co-digestion of distiller's grains and food waste. Biosyst. Eng. 112, 130–137. doi:10.1016/j.biosystemseng.2012.03.006

Ward, A.J., Hobbs, P.J., Holliman, P.J., Jones, D.L., 2008. Optimisation of the anaerobic digestion of agricultural resources. Bioresour. Technol. 99, 7928–40. doi:10.1016/j.biortech.2008.02.044

WEF, 2018. Biogas data [WWW Document]. URL http://www.resourcerecoverydata.org/biogasdata.php

Won, S.G., Ra, C.S., 2011. Biological nitrogen removal with a real-time control strategy using moving slope changes of pH(mV)- and ORP-time profiles. Water Res. 45, 171–178. doi:10.1016/j.watres.2010.08.030

Xu, S., Selvam, A., Wong, J.W.C., 2014a. Optimization of micro-aeration intensity in acidogenic reactor of a two-phase anaerobic digester treating food waste. Waste Manag. 34, 363–369. doi:10.1016/j.wasman.2013.10.038

Xu, S., Selvam, A., Wong, J.W.C., 2014b. Optimization of micro-aeration intensity in acidogenic reactor of a two-phase anaerobic digester treating food waste. Waste Manag. 34, 363–369. doi:10.1016/j.wasman.2013.10.038

Yamamoto, E., Muramatsu, H., Nagai, K., 2014. *Vulgatibacter incomptus* gen. nov., sp. nov. and *Labilithrix luteola* gen. nov., sp. nov., two myxobacteria isolated from soil in Yakushima Island, and the description of *Vulgatibacteraceae* fam. nov., *Labilitrichaceae* fam. nov. a. Int. J. Syst. Evol. Microbiol. 64, 3360–3368. doi:10.1099/ijs.0.063198-0

Yin, J., Yu, X., Zhang, Y., Shen, D., Wang, M., Long, Y., Chen, T., 2016. Enhancement of acidogenic fermentation for volatile fatty acid production from food waste: Effect of redox potential and inoculum. Bioresour. Technol. 216, 996–1003. doi:10.1016/j.biortech.2016.06.053

Zhou, W., Imai, T., Ukita, M., Li, F., Yuasa, A., 2007. Effect of limited aeration on the anaerobic treatment of evaporator condensate from a sulfite pulp mill. Chemosphere 66, 924–929. doi:10.1016/j.chemosphere.2006.06.004

Zhu, M., Lü, F., Hao, L.-P., He, P.-J., Shao, L.-M., 2009. Regulating the hydrolysis of organic wastes by micro-aeration and effluent recirculation. Waste Manag. 29, 2042–50. doi:10.1016/j.wasman.2008.12.023

Zitomer, D., Maki, J., Venkiteshwaran, K., Bocher, B., 2016. Relating Anaerobic Digestion Microbial Community and Process Function. Microbiol. Insights 8, 37. doi:10.4137/MBI.S33593

Zitomer, D.H., Shrout, J.D., 1998. Feasibility and benefits of methanogenesis under oxygen-limited conditions 18, 107–116.